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The kinetics of the hemolysin of Newcastle disease virus

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THE KINETICS OF THE HEMOLYSIS OF NEWCASTLE DISEASE VIRUS

by

Edward Michael Faddoul

**A Dissertation Submitted to the
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INTRODUCTION

Research interest in Newcastle disease virus stems not only from its pathogenicity but from its serological properties as well. The serological properties: immune reactions, hemagglutinating activity and hemolytic activity are useful tools in the study of the nature of viruses and their interaction with host cells. Of these serological properties hemolytic activity, the alteration of the structural integrity of the red blood cell causing it to leak its hemoglobin into the solution, is particularly interesting as a possible reflection of the mechanism by which viruses invade or leave host cells. An understanding of the details of viral hemolysis might provide valuable clues to the mechanism of virus infection.

Newcastle disease virus is one of several, but not all, of the myxoviruses that exhibit hemolytic activity. This property is not very evident in these viruses as they are harvested in the fluids of infected chicken egg embryos but it may be greatly enhanced by chemical and physical techniques which leave other properties of the virus essentially unaffected. During purification procedures, the hemolytic activity migrates with the intact virus. This suggests that hemolysis is caused by patches or sites on the virus surface and not by free hemolysins released from the virus. It is thought that the enhancement procedures unmask hemolytic sites on the surface of the virus either by inducing some change in the virus itself or by effecting the removal of hemolytic inhibitors.

In general, the hemolytic action of Newcastle disease virus appears to be enzymatic in nature and closely associated with the hemagglutinating

structures. Red blood cells which are immune to hemagglutination are also immune to hemolysis. The hemolytic activity is heat labile, is inhibited by calcium and exhibits pH and temperature optima. The hemolytic property has exhibited enhancement and inhibition responses to several chemical agents commonly regarded as enzyme activators or inhibitors.

The search for a discrete, isolatable product of the hemolytic reaction other than hemoglobin has so far been unsuccessful. Some destruction of sphingomyelin in chicken erythrocyte stroma has been noted as accompanying viral hemolysis but further study showed that these events are not directly related. At present, therefore, studies on the nature of Newcastle disease virus hemolysin are limited to those things which can be learned from virus-red blood cell interaction.

A potentially fruitful approach to an investigation of virus-red blood cell interaction is the measurement and interpretation of reaction kinetics. From such data it is possible to suggest probable reaction mechanisms which may in turn hasten the identification of the hemolytic unit. The kinetics of enzyme-moderated reactions have been studied extensively and the theoretical interpretation of these data is well grounded.

Some previous attempts to measure and interpret the kinetics of viral hemolysis have been reported. An important fact brought to light by these efforts is that the action of the viral hemolysins is very rapid, apparently being much more rapid than the observable appearance of hemoglobin. The speed of viral attack was such that virtually all of the hemolysis kinetics data was collected during the later period of hemoglobin loss.

The interpretation of these data, consequently, may not provide adequate information on which to base a hypothesis of the kinetics of viral hemolytic attack on an erythrocyte.

Much could be learned about the hemolytic virus-red blood cell interaction from the initial kinetics of the reaction. It is proposed that the hemolytic reaction be studied for the purpose of obtaining initial kinetics data. If necessary, techniques appropriate to obtain these data will be developed. The initial kinetics data will be utilized in an attempt to interpret the virus-red blood cell interaction mechanism in terms of a classic enzyme-substrate interaction. If such an interpretation is possible it will lend further support to the hypothesis that viral hemolysis is enzymatic in nature and may permit some inferences to be drawn concerning the nature of the hemolytic unit itself.

REVIEW OF THE LITERATURE

Newcastle Disease Virus

Newcastle disease virus (NDV) is an avian respiratory pathogen which is found on several continents and exists in a number of distinguishable strains. NDV was first isolated and identified more than 40 years ago and has been studied extensively by workers representing a variety of scientific disciplines. Initially, the purpose of these studies was to reduce its economic impact upon the poultry industry. The in vitro properties of this virus, however, provide an additional and continuing challenge to scientific investigation which may be remote from pathogenic or economic considerations per se. This in vitro behavior of the virus along with its chemical and physical properties now serves as the primary basis for the taxonomic classification of NDV among the known viruses [16, 19, 50]. Under the scheme of this classification, NDV is a myxovirus, one of a group of viruses which includes human influenzae: A, B, and C, fowl plague, mumps and the para-influenzae, including para-influenza 1 (Hemagglutinating Virus of Japan, HVJ, or Sendai). The system of classification is in some degree of flux due to a rapidly expanding knowledge of viral morphology, chemistry, physics and serology. Consequently, the above list of viruses does not constitute the entire group Myxovirus but their long-recognized association and universal acceptance as members of this group [1, 2, 11, 24, 27, 35, 44] provides firm ground for expecting these viruses to continue to be grouped together in future classification systems.

Newcastle disease virus is usually propagated in the laboratory in chicken embryos by inoculation into the allantoic cavity some 10 to 12

days after the eggs have been set. Most of the embryos die during the next 48 hours after which the allantoic fluid is harvested to obtain the virus in high titer [4, 13, 45, 49]. The virus in crude allantoic fluid is roughly spherical in shape with a diameter of 80 - 120 m μ and a molecular weight of approximately 500,000 [6, 7, 8]. This allantoic fluid may then be treated in a variety of ways to obtain suitable virus preparations.

Frequently, the infected allantoic fluid from a number of embryos is pooled to obtain a supply of virus material sufficient for a number of experiments. The pooled harvest offers uniformity of samples and greater convenience in handling. It is often practical to divide the pool into portions appropriate to experimental needs and to store these portions until required. The most common means of purification, concentration and preservation of the virus include dialysis, ultracentrifugation, lyophilization, and freezing. Experience with other biological materials indicates that these are relatively gentle methods, but even these may cause certain observable changes in the virus particles. The virus population may undergo an alteration in appearance to electron microscopy, a reduction in the degree of association or aggregation and, often, some loss in infectivity titer.

Bang [6, 7, 8] spun down the virus from the allantoic fluid and re-suspended it in either distilled water or in saline solutions of 0.07 to 0.15 molar. The spherical forms present in the allantoic fluid were also found in the distilled water suspension but appeared to undergo a conversion to filamentous, sperm-like forms in saline. Both forms exhibited equivalent infectivity titer and reaction with immune sera. The conversion ap-

peared to be reversible. Similar results were obtained when infected allantoic fluid was dialyzed against saline solution.

Bang [8] observed further that freezing and thawing the infected allantoic fluid causes approximately a 10-fold increase in the infectivity titer and in hemagglutinin activity in the top layer of the 30,000 g centrifugate supernatant. This may be attributed either to a viscosity increase of the suspending medium or to a disintegration or disaggregation of the virus particles themselves. This finding must not be confused with the work of Hirst et al. [33] and of Lucie [49] who reported the removal of large amounts of active virus by the low-speed sedimentation of slow-thawed allantoic fluid precipitate (2500 g or less). In the former case the centrifugal acceleration is sufficient to sediment individual virus particles, in the latter the acceleration is too weak to sediment any virus except that occluded in or adsorbed to a precipitate.

NDV-Erythrocyte Interaction

Hemagglutination

One of the most distinctive properties of NDV and one which associates it with the other myxoviruses is hemagglutinating activity (HA) [2]. Viruses exhibiting HA attach to certain erythrocytes which then aggregate into di- and multi-cellular clumps which settle more rapidly than the individual cells [45]. The individual cells settling in a test tube will slide down the concave sides of the bottom to the lowest point and there collect in a compact "button" of cells. Agglutinated cells to the contrary, do not slide appreciably but remain where they fall in a diffuse pattern covering the bottom of the test tube [45]. After a period of time,

the virus elutes from the erythrocytes and the clumps of cells disaggregate. Thereafter, the cells if resuspended will settle in the normal pattern for non-agglutinated cells. They are, however, immune to reagglutination by the addition of fresh virus of the same strain. The chemistry of the HA phenomenon has been investigated by many workers [11, 12, 15, 18, 27, 28, 29, 30, 41, 45, 48, 57] who have amassed overwhelming evidence that at least the virus elution phase of HA is enzymatic in nature.

Typical of enzymatic reactions, viruses exhibiting HA can be shown to exhibit substrate specificity: avian, human, and guinea pig erythrocytes are agglutinated by all established myxoviruses; rabbit erythrocytes are totally resistant to agglutination [14, 18]. The reaction is temperature dependent and pH dependent [18, 27, 69] and the existence of immunologic inhibitors [27] has been demonstrated. There are no significant cationic inhibitors to HA but Gardner and Morgan [30] have shown that reducing agents such as glutathione and cysteine markedly enhance the HA of mumps virus. The HA reaction is irreversible; after a time the virus elutes from the erythrocyte leaving red blood cells which are immune or "stabilized" to subsequent agglutination by a fresh inoculation of the same virus strain. The eluted virus, however, remains fully active toward fresh erythrocytes. The HA attachment mechanism appears to utilize the same erythrocyte attachment sites regardless of the particular myxovirus under study [15]. Burnet [13] and Burnet et al. [15] have demonstrated a linear gradient of viral HA affinity for the susceptible erythrocyte. Myxoviruses higher in the series can agglutinate red blood cells previously stabilized to myxoviruses lower in the series. Erythrocytes agglutinated by myxoviruses

high in the series are stabilized to the HA of all myxoviruses lower in the series. Gottschalk [31] in 1957 adequately reviewed the subject of virus enzymes and discussed at length the involvement of influenza virus neuraminidase (sialidase) in the overall hemagglutination reaction.

The work with neuraminidase has been continued and extended into the most recent literature where Seto et al. published in 1966 a method for the isolation of neuraminidase from influenza virus [72]. Seto, Drzeniek and Rott utilized cold butanol extraction followed by ether extraction of a dialyzed, lyophilized influenza virus concentrate to obtain a residue susceptible to attack by pronase. The soluble enzyme released by the pronase hydrolysis was isolated by ultracentrifugation and column chromatography. This protein isolate exhibited a potent neuraminidase activity. Drzeniek and co-workers [24] continued the study of these viral neuraminidases by isolating the enzyme from fowl plague virus and from NDV. They have demonstrated that the influenza virus and fowl plague virus neuraminidase isolates are free of other antigenic properties and that some differences exist among the chemical properties of the three isolates.

The existence of an enzyme associated with hemagglutination implies the existence of a discrete substrate on or in the erythrocyte. The characterization of the enzyme as a neuraminidase has proceeded in parallel with the identification of the substrate. Gottschalk [31] has presented evidence to show that neuramin-lactose is a synthetic substrate for neuraminidase. "The action of 'neuraminidase' is defined as the hydrolytic cleavage of the glycosidic linkage joining the keto group of neuraminic acid to D-galactose or to D-galactosamine or possibly to other sugars..." - Gottschalk [31].

Kathan and co-workers [41, 42, 43] have succeeded in isolating from human erythrocytes a glycoprotein, molecular weight approximately 31,000, containing about 50% carbohydrate and exhibiting an intense myxovirus HA inhibitor activity. They believe that this glycoprotein, homogeneous by paper and moving boundary electrophoresis, ultracentrifugation, column chromatography and end group analysis, is identical with the receptor site of erythrocytes [41]. Amino acid analysis of the protein portion of the glycoprotein indicates that there are 16 amino acids present, of which serine and threonine are most abundant and aspartic acid seems to be located at the NH_2 - terminus of the chain. There appear to be 23 moles of sialic (diacetylneuraminic) acid per 31,000 grams of glycoprotein. These residues may be linked via a disaccharide or a trisaccharide to the serine or threonine of the protein chain. All of the sialic acid appears to be in a terminal position since it can be totally removed by the action of influenza virus PR-8 [41]. It is interesting to note that Asian influenza virus did not remove all of the sialic acid and that these authors have demonstrated that the Asian strain of influenza virus is lower in the linear gradient of HA as defined by Burnet and co-workers [15]. This finding suggests that the myxoviruses have varying efficiency in the removal of sialic acid residues from the receptor site.

There seems to be more involved in the overall process of hemagglutination (virus attachment, enzyme action, virus elution) than just an enzymatic attack on the sialic acid residues of the erythrocyte receptor sites, i.e., the virus HA structure contains more than a single functional specie, a neuraminidase. Evidence in this direction begins with several

published studies on the effect of organic solvents and of periodate ion on some of the myxoviruses and on the receptor sites for virus attachment to a host cell or to an erythrocyte. Fazekas de St. Groth [25] and Fazekas de St. Groth and Graham [26] published a broad study of the effect of IO_4^- ion on influenza virus, on host cells, and on erythrocytes. In summary, they observed that periodate in appropriate concentration modifies the receptor sites on erythrocytes so that influenza virus attaches to the treated cells and agglutinates them but will not elute nor can it be removed with receptor destroying enzyme (V. cholerae neuraminidase). The same concentration of periodate used to pretreat the virus has no effect on either HA titer or on infectivity titer. Host cells treated with periodate before addition of virus are infected to endpoints identical with untreated host cells [26]. Chu and Morgan [18] also studied the effect of periodate on mumps virus and on chicken erythrocytes. In 1952 Hoyle [40] utilized the known disruptive effect of diethyl ether on influenza virus to carry the study of virus agglutinin - periodate-treated-erythrocyte one step farther. Ether disrupts influenza virus into fragments each of which has distinctive composition and serological properties [22, 46]. One of these fragments displays full HA including the ability to destroy the receptor sites (neuraminidase hydrolysis). Hoyle found that periodate oxidation of this fragment destroyed the neuraminidase activity but did not affect the attachment of the particle to the erythrocyte receptor. This refinement of the prior work with intact viruses tends to support the concept that the hemagglutinating unit on the normal virus contains at least two distinct functional elements: an attachment mechanism and a neuraminidase [40]. Conversely, existing evidence suggests that the re-

ceptor site contains only one type of necessary chemical structure: one or more terminal-position glycosidyl-sialic acid residues.

Hemolysis

Nature of hemolysis In addition to hemagglutinating activity, some of the myxoviruses have been shown to possess the ability to lyse erythrocytes. Hemolysis, the lysis of red blood cells, is any process by which the hemoglobin normally contained within each cell is lost to the suspending medium. This event may occur with or without visible (in the light microscope) changes in the appearance or integrity of the erythrocyte cell membrane (stomata) [54, 59, 60]. In general, it may be said that considerations of HA phenomena, such as those presented in the preceding paragraphs, are concerned with interactions between hemagglutinins and red blood cell surface structures. Hemolytic phenomena involve not only erythrocyte surface changes but also conditions and properties throughout the interior of the cell.

In recent years the erythrocyte has been found to be a much more complex structure than originally supposed, a complexity which demands an extremely sophisticated model to describe accurately the mechanism of hemolysis. Much effort has been devoted to a study of the surface changes resulting from the action of various hemolysins [10, 21, 65, 66, 68]. The actual mechanism by which, after action of a hemolysin, the hemoglobin passes from the interior of the erythrocyte to the suspending medium remains obscure. It would appear that there are several possible mechanisms involved; the one that occurs depends upon the hemolysin and a number of other variables. Ponder's 1948 monograph [60] provides an excellent dis-

cussion of the entire subject of hemolysis in the mammalian (non-nucleated) red cell. An appendix is devoted to such specific details as were available concerning hemolysis in the nucleated erythrocyte.

In the monograph just cited, Ponder explained the sigmoid character of hemolysis - versus - time curves as the logical consequence of heterogeneous red blood cell resistance to the hemolysin. A later paper by Ponder and Cox [61], in response to critics of the heterogeneous-resistance explanation, demonstrates that there is, indeed, a distribution of erythrocyte resistances to (at least) the lysins tried. A further report of some importance in this same paper is a study of "the type of the hemolytic reaction" [61]. Using simple but effective experiments, the authors show that hemolysis, once initiated by a lysin, is slowed down but not stopped by the removal or dilution of the lysin. This implies that hemolysis is not "...the immediate effect of the capture of lysin by a cell." In their own words:

To explain this it seems necessary to suppose that the interaction between the lysin and the cell, although it may be reversible in respect to the alteration of the lysin is, in its effect on the cell, so far from being reversible that it is not even adequately described as irreversible. The characteristic of the process taking place in the cell is that, once it is well enough under way, it cannot be stopped by diluting the lysin in the system. A reaction of this kind can be called a progressive reaction. [61] (*Italics theirs.*)

Virtually all of this work on hemolytic phenomena had been based upon hemolysis induced by "simple" hemolysins (chemical compounds such as saponins, detergents, bile acids, triphenylmethane dyes, lysolecithin, etc.) or physical stress such as osmotic shock, freeze-thaw, ultrasonics, maceration, etc. Perhaps a notable exception to this generalization is the rather extensive literature concerned with the hemolysis induced by

the action of immune sera [10]. Very little research has been reported on hemolysis induced by the direct action of purified enzymes upon erythrocytes. Ponder [60] mentions that lipases do not cause hemolysis but that papain and mexacain hemolyze human cells. Gardner and Morgan [30] demonstrated that lecithinase A is a hemolysin active on chicken erythrocytes.

Viral hemolysins Historically, the first evidence of a viral hemolysin was published in 1948 by Morgan et al. [56] as a consequence of their studies on mumps virus. It is significant to note that in this first report they recognized several enzyme-like properties of the hemolytic activity. Later work has confirmed and extended their observations. Within a year the phenomenon had also been observed in NDV-erythrocyte interaction [44] and since has been found in studies with Sendai virus (HVJ) [35], para-influenza 3 and measles viruses [1]. The influenza viruses have so far failed to exhibit any detectable hemolytic effect. By contrast, the hemolytic viruses are now sufficiently well characterized that their hemolytic properties are readily demonstrated if not well understood. These hemolytic properties of mumps virus, Sendai virus and NDV have been found to be very similar, so much so that it is often possible to discuss their characteristics as a group. For the purpose of greater clarity of presentation hereafter, NDV, mumps virus and Sendai virus will be referred to collectively as "the hemolytic viruses".

Typically the hemolytic viruses show little or no hemolytic activity in their native state, i.e., in freshly harvested embryonic fluids [13, 14, 17, 32, 35, 48, 49, 53]. Almost any treatment, even as mild as mere dilution with phosphate buffered saline [14, 32], will enhance the hemolytic

activity of these viruses. Burnet and Lind [14] showed that methanol-precipitated NDV, resuspended in physiological saline, exhibits much greater hemolytic activity than an equivalent HA titer of untreated virus. Chu and Morgan [17] studied mumps virus to observe that freezing and thawing infected allantoic fluids greatly enhanced hemolytic activity without changing the hemagglutination titer. Granoff and Henle [32] studied the effect of dialysis, osmotic shock, freezing and thawing, and sonic vibration on NDV in allantoic fluid. All of these stresses enhanced the hemolytic activity with little or no change in HA titer. Hosaka [35] used a centrifugally separated fraction of Sendai virus resuspended in phosphate buffered saline for his studies on agents influencing hemolytic activity. His findings were essentially identical to those previously cited: freeze-thaw, osmotic shock, slow dilution, and sonic vibration all enhance hemolytic activity. Wilson [76] treated NDV-infected allantoic fluid with a fluorocarbon solvent (Genetron 113) and observed a 10-fold increase of the hemolytic titer.

These studies on factors enhancing the hemolytic activity of NDV, mumps virus and Sendai virus were reviewed recently by Lucie [49]. She observed that there were wide variations in the degree of enhancement obtained and postulated that this might be explained in part by variations in technique and by actual virus strain differences. Her studies with NDV confirmed that both technique and virus strain employed are important factors controlling the degree of enhancement. One of the most significant variables of technique was found to be the thawing procedure of the freeze-thaw enhancement of hemolytic activity. She demonstrated that the techniques could be improved to provide repeatable and reproducible

enhancement of hemolytic titer.

Enzymatic characteristics of viral hemolysins It was mentioned briefly that Morgan and co-workers [56] noted enzyme-like characteristics in their first report of a viral hemolysin (mumps). The successful enhancement of hemolytic activity provided many workers with the opportunity to study the influence of physical and chemical stress on these viral hemolysins. The response of the hemolysins to stress conditions may provide evidence upon which the hypothesis that they are enzymes may be judged.

Heating the hemolytic viruses at temperatures in the range 45° - 50°C destroys the hemolytic activity. Often it is possible to select a temperature and time which will permit inactivation of the hemolysis while leaving HA and infectivity little affected [13, 17, 36, 53, 56, 70].

Calcium has been shown to be a potent inhibitor of viral hemolysis [14, 36, 53, 55, 70] but has no inhibiting effect on HA [48]. Morgan [55] also studied manganese and magnesium as inhibitors of hemolysis but found that their effect is much less than that of calcium. His studies suggest that the inhibitory properties of allantoic fluid (either normal or infected) are principally due to the presence of calcium. He obtained a large increase of hemolytic titer by flowing allantoic fluid over a cation exchange resin.

Gardner and Morgan [30] studied the inhibitory effect of other chemicals which were known to inhibit various enzymes. They found mumps virus hemolysin to be inhibited by urethane, hydroxylamine hydrochloride and glutathione. Cysteine enhances both hemolytic and hemagglutinating activity. Similar studies by Morimoto and Morgan [57], with mumps virus,

showed that pretreatment of erythrocytes with lecithin, cholesterol and cardiolipin reduced the hemolytic activity. The effect of these compounds was additive, producing more inhibition in combination. There was no inhibition if the compounds were added after mixing erythrocytes and virus.

It has been shown quite generally that pretreatment of any of the hemolytic viruses with homologous immune serum prevents hemolysis [14, 34, 44, 48, 53, 56, 70, 75]. Also, pretreatment of the red blood cells with receptor destroying enzyme (RDE) or with such non-hemolytic myxoviruses as influenza PR8 prevents hemolysis by the hemolytic viruses [14, 18, 53, 70]. These agents are known to prevent HA [11, 15, 27] and this suggests that a virus-red blood cell attachment of the hemagglutinative type is required before hemolysis can occur. This hypothesis is supported further by studies with red blood cells from various species.

Burnet and Lind [14] and Chu and Morgan [18] showed that erythrocytes resistant to agglutination (rabbit red blood cells in particular) were also resistant to hemolysis. Sato [70] and Chu and Morgan [18] pretreated susceptible erythrocytes with periodate, which was known to destroy red cell HA receptors [25, 26], and again observed that destruction of HA receptors also prevented hemolysis.

The hemolytic activity of the hemolytic viruses exhibits temperature and pH dependence. Many workers have shown that hemolysis is greatly inhibited if the temperature of the virus-red blood cell mixture is kept below 15°C [14, 35, 49, 53, 56, 70]. This characteristic appears to be universal with these viruses and is specific to the hemolysin since the HA-type of attachment proceeds in the cold. This property has been used by a number of authors as a part of the experimental regimen in the study

of hemolysis. Lucie [49], however, suggests that pre-incubation of hemolytic NDV with erythrocytes in the cold may cause variation in the repeatability of hemolysis assays. The ideal temperature for viral hemolysis appears to be in the range $35^{\circ} - 39^{\circ}\text{C}$ [35, 49, 69].

Analagous to the optimum temperature range, there is an optimum pH range for hemolysis. The pH - activity profile shows that maximal hemolytic activity is obtained when the pH is held in the range 6.5 - 7.5 [18, 35, 49, 56]. A detailed study of the temperature, pH, and virus:erythrocyte ratio influence on the hemolytic activity and HA of NDV has been published by Sagik and Levine [69].

The effect of storage temperature on hemolysin survival has also been studied. Non-frozen cold storage preserves hemolytic activity for varying lengths of time (from 10 days to 6 weeks) depending on the temperature and the type of virus. Frozen storage at -20°C extends the survival of hemolysin to at least several months [17, 53].

Hosaka [36, 37] utilized hemolytic NDV and Sendai virus purified by ultracentrifugation to study the products of erythrocyte hemolysis. He observed a decrease in sphingomyelin content of chicken erythrocyte stroma which was related to the hemolytic activity of the viruses. He was unable to show, however, that this destruction was necessary for hemolysis since a similar effect was not observed during hemolysis of either human or guinea pig erythrocytes. Furthermore, there was no attack by these viruses on sphingomyelin purified from chicken erythrocytes. These findings are very similar to those of Moberly et al. [54] and Soule and co-workers [73] working with hemolytic mumps virus. They also observed a decrease of sphingomyelin in chicken erythrocyte stroma but no change in sphingomyelin content

of human red blood cell stroma lysed by the virus. None of these studies revealed a reaction product of the hemolytic attack other than the hemoglobin released. In view of the findings of these experiments it is improbable that protein-bound sphingomyelin is the primary point of attack of the viral hemolysins.

The many enzyme-like properties of the hemolytic activity of mumps virus, NDV and Sendai virus have prompted most of the workers cited above to comment that viral hemolysins appear to be enzymatic in nature. They have demonstrated that the hemolytic activity is heat labile, is temperature and pH dependent, is subject to calcium ion inhibition, is inactive toward erythrocytes immune to HA, and is enhanced by various purification procedures. They have demonstrated further that there are several chemical and physical inactivating agents other than heat: ether [35], formalin [17, 35, 53, 70], ultraviolet radiation [17, 70] and ionizing radiation [74, 75]. Taken collectively, these properties present a fairly strong circumstantial case favoring the hypothesis that the hemolysin unit is an enzyme.

Matsumoto et al. [52] and Matsumoto and Maeno [51] discovered a phenomenon which, although not negating the hemolytic enzyme hypothesis, must eventually be explained by a satisfactory theory of viral hemolysis. They observed that Sendai virus exhibits different infective and serological behavior when it is propagated in mice than when it is propagated in egg embryos. The hemolytic activity of Sendai virus grown repeatedly in egg embryos was lost after one passage in mouse tissue. Non-hemolytic Sendai virus from mouse tissue culture became hemolytic after a single

passage in egg embryos. The hemagglutinating properties of the virus also changed with the change in host cell but the alteration was more gradual, requiring several passages to come to completion. The authors suggest that these observations imply a host-induced alteration in the virus particle. Drake and Lay [23] observed a host-induced variation in the response of NDV infectivity to inactivating agents such as heat and ultraviolet light. Their studies did not include an evaluation of the effect of change-of-host on the hemolytic activity of this virus.

Hemolysin identification efforts In conjunction with the efforts already cited there have been several attempts to isolate the hemolysin responsible for the activity of these viruses. When hemolytic virus, adsorbed to red blood cells in the cold, is eluted the hemolytic activity and HA titer of the eluate rise simultaneously [35, 44, 56, 70]. Differential centrifugation of the infected allantoic fluids has been carried out and the various fractions have been tested for infectivity, HA and hemolytic activity. In every case the hemolytic activity has been associated with the fraction identified as containing the intact virus particles [32, 35, 49]. Schmidt et al. [71] and Wilson [77] used DEAE columns to purify NDV and obtained similar fractionation patterns. Only Schmidt and co-workers reported on the fate of the hemolytic activity which was not separated from the HA by this technique.

Atanasiu et al. [4, 5] have claimed success in the effort to separate a hemolysin subunit from the intact virus particle. They used a pressure cell to subject NDV to 4000 kg/cm^2 and centrifuged the pressure-treated material at $20,000 \text{ g}$ for 90 minutes. The supernatant exhibited high

hemolytic titer and low HA and no infectivity. The sediment exhibited only HA titer. Although they suggest that a hemolysin may have been liberated from the virus, it is also possible that other interpretations are equally valid. The references previously cited indicate that a hemagglutination-type virus-erythrocyte interaction is necessary for hemolysis to occur. This observation implies that an HA unit, at least, was associated with the hemolytic units isolated by Atanasiu and co-workers. HA unit-containing fragments of the virus particles could attach to the erythrocyte receptors without yielding a positive agglutination since they would be unable to cause the cells to clump. Furthermore, Lucie [49] has shown that an appreciable number of infective units ($L.D._{50} = 10^{-6.5}$) remain suspended after 60 minutes of centrifugation at 28,000 g. This implies that particles the size of intact virus may have been present in the supernatant of Atanasiu et al. [4, 5]. These considerations suggest that there may be acceptable alternatives to their conclusion that they had succeeded in releasing a free viral hemolysin unit.

Another effort to identify the hemolytic unit was published by Rebel et al. [62]. They chromatographed the phospholipids of Sendai virus-infected allantoic fluid, normal allantoic fluid and extracts of Sendai virus. On the basis of these findings and the fact that methanol-precipitation of Sendai virus destroys its hemolytic activity, they concluded that the hemolytic unit is lysolecithin adsorbed on the surface of the virus particle. This hypothesis is difficult to accept in the face of the known calcium inhibition of Sendai virus-induced hemolysis.

Lysolecithin forms complexes with such divalent ions as cadmium and zinc but these are also hemolytic. Furthermore, lysolecithin is stable to temperatures in excess of 100°C whereas the hemolytic activity of Sendai is destroyed at 45°C. It would appear that their hypothesis is premature.

Hemolysin concentration The effect of lipid solvents on myxo-viruses has been studied extensively [3, 22, 38, 39, 46, 67] for the purpose of structural analysis, composition analysis, and the preparation of vaccines. It was found that methanol and ethanol could be used to purify and concentrate these viruses [20, 28, 33]. Burnet and Lind [14] used methanol precipitation to obtain a hemolytic preparation of NDV which they used for their studies on the hemolytic properties of this virus. Wilson [76] showed that trichlorotrifluoroethane extraction of NDV also increased hemolytic activity. On the other hand, Soule et al. [73] and Sato [70] found that methanol precipitation of mumps virus and of Sendai virus destroyed the hemolytic activity. The cause of these two opposing properties on otherwise similar viruses remains obscure.

There is another method for the purification of hemolytic NDV which works well, yielding virus with high hemolytic activity, infective titer and HA titer. Lucie [49] observed that a precipitate formed when frozen normal or infected allantoic fluid was thawed at 4°C. The precipitate, separated from the bulk of the fluid by centrifugation and decantation, was taken up in citrated physiological saline at 37°C. Approximately half the infective units present in the original material was associated with this precipitate fraction. Although the infectivity of the precipitate was equivalent to that of the decantate, the hemolytic activity was very

much greater. Further stress on the reconstituted precipitate was unable to increase the hemolytic activity further. Lucie postulated that the hemolytic sites on this virus were fully activated by the initial freeze-thaw treatment. She was unsuccessful in her efforts to identify the agent which caused the hemolytic virus to be selectively associated with the precipitate of the infected allantoic fluid.

Hemolysis Kinetics

With the possible exception of Atanasiu et al. [4, 5], no one has yet succeeded in isolating a hemolytic fragment distinct from the virus particle. This has hampered definitive work on the identification of the hemolysin or the elucidation of the hemolytic mechanism. It is possible to obtain some information concerning the mechanism of hemolysis by studying the kinetics of the reaction. Ponder [60] has discussed hemolysis kinetics emphasizing the point of view of the change taking place in the erythrocyte during this process. Bernheimer [9] has published a classification of several bacterial and chemical hemolysins based upon the kinetics of their interaction with erythrocytes. Bowman and co-workers [10] studied the kinetics of immune hemolysis to show that the erythrocyte-antibody combination is reversible and that the antibody can migrate from cell to cell during hemolysis. These workers had the advantage of working with a well-defined hemolysin which could be measured independently and at least reasonably quantitatively. It is not yet possible to define the hemolytic activity of hemolytic viruses by independent criteria. Lucie [49], in particular, observed that there is no correlation between in-

fectivity and hemolytic activity and little, if any correlation between HA and hemolytic activity. All other workers in this field have encountered the same problem. Those who have tried to measure the kinetics of viral hemolysis have used hemolytic virus preparations identical to those used in their other studies.

Burnet and Lind [14] observed that receptor destroying enzyme (RDE) did not significantly inhibit hemolysis when added to the system only 10 seconds after mixing virus and erythrocytes. The stabilizing action of RDE is fairly rapid since red blood cells treated with RDE for one minute before adding virus exhibit substantially reduced hemolysis. RDE acting on erythrocytes destroys the HA receptor sites which are required for both hemagglutination and hemolysis. If virus is attached to these sites prior to the addition of RDE, the virus is not removed by the enzyme but elutes normally. The observations of Burnet and Lind concerning the effect of RDE on hemolysis suggest that much of the viral attachment process is complete within ten seconds after mixing virus and red blood cells. Neither Burnet and Lind [14] nor Hosaka [35] were able to stop the hemolytic reaction by chilling after 6-7 minutes at 37°C. These authors as well as Granoff and Henle [32] obtained similar rate curves for viral hemolysis. Hosaka [35] has interpreted these as indicating a reaction first order in substrate (red blood cell) concentration. Since all the data was gathered in the time interval during which hemolysis was known to proceed spontaneously (i.e., 6 minutes or more after initiation of hemolysis) this first order behavior is not surprising. There is some question, however, as to whether these kinetics are of the virus-erythrocyte interaction or

of hemolytic events independent of it.

Lineweaver and Burk [47] demonstrated that enzymes which follow the mechanism



yield kinetics data such that the reciprocal of the reaction rate (velocity) is a linear function of the reciprocal of the substrate concentration.

This relationship provides the familiar Lineweaver-Burk reciprocal plot of $1/v$ vs. $1/S$. The relationship is linear only for mechanisms of the type illustrated and kinetics data obtained in the course of enzyme research do not necessarily exhibit this linear relationship. The interpretation of mechanism from such data is more complicated than the illustration above. Reiner [64] has explained in detail effective methods to treat kinetics data which result from more complicated enzyme mechanisms.

MATERIALS AND METHODS

Reagents

Alsever's solution:

The Alsever's solution was prepared according to the recipe:

20.5 g Dextrose ($C_6H_{12}O_6$)

8.0 g Sodium Citrate ($Na_3C_6H_5O_7 \cdot 2H_2O$)

4.2 g Sodium Chloride ($NaCl$)

These reagents were diluted to one liter with deionized distilled water. The pH was adjusted to 7.4 with 1N sodium hydroxide. The final solution was sterilized by filtration through an O2 Selas filter candle, portioned into sterile bottles, and stored under refrigeration.

Cox's buffer:

The reagents

17.3 g Sodium Phosphate, Dibasic ($Na_2HPO_4 \cdot 7H_2O$)

4.6 g Sodium Phosphate, Monobasic ($NaH_2PO_4 \cdot H_2O$)

were dissolved in deionized distilled water and diluted to one liter. Adjustment to pH 7.0 was accomplished by addition of either 1N H_3PO_4 or 1N NaOH as the case required. The solution was sterilized by filtration through an O2 Selas filter candle, portioned into 50 ml sterile bottles, and stored under refrigeration.

Saponin:

The saponin used in some experiments for total hemolysis was obtained as the purified powder from Fisher Scientific Co.¹

¹Fisher Scientific Co., 1458 N. Lamon Ave., Chicago, Ill., 60651

Citrate-Saline:

The citrate-saline was prepared by dilution of

8.5 g Sodium Chloride (NaCl)

20.0 g Sodium Citrate ($\text{Na}_3\text{C}_6\text{H}_5\text{O}_7 \cdot 2\text{H}_2\text{O}$)

with deionized distilled water to one liter. The pH was adjusted to 7.4 by addition of Cox's buffer. This solution was usually prepared in large quantity and stored in the refrigerator. When sterile citrate-saline was required, the above solution was autoclaved in a two-liter erlynmeyer flask with sidearm. The sidearm vent was protected with a cotton plug. The sterile solution was dispensed using an attached 5 ml Cornwall pipettor. The sterility of the tip of the pipettor was protected with an alcohol swab wrapping during storage.

"Mix"

A combination of two antibiotics,

5,000 units Penicillin G¹

1.0 g Streptomycin Sulfate¹

dissolved and diluted to 10 ml with deionized distilled water was used to protect NDV infectivity titrations from chance contamination with bacteria. This combination was designated "Mix" and was added to the fluids for titration as a part of the diluent. The ratio routinely used was 10% "Mix" by volume.

¹ Eli Lilly Co., Indianapolis, Indiana

Virus

A substrain, designated 174HS, of the parent strain 174 Iowa, was obtained from stocks maintained at the Veterinary Medical Research Institute. This choice of virus population was based upon information concerning the hemolytic behavior of several substrains of NDV as reported by Lucie [49]. In particular, NDV substrain 174HS was found to exhibit a relatively high titer of hemolytic activity when subjected to proper treatment.

This substrain, 174HS, of 174 Iowa has been previously described in the literature (Picken [58]). Briefly, the substrain was obtained from the parent stock by a thermal selection process. This particular substrain is a population of which the HA property is heat-labile to the extent that it is destroyed within ten minutes at 56°C. Another substrain, 174HR, from the same parent stock has been selected on the basis that its HA is heat-resistant for at least 60 minutes at 56°C.

All of the virus, 174HS, used in this study was prepared from infected chicken embryo allantoic fluids. Ten-day-old chicken embryos were inoculated with 0.1 ml of a 10^{-4} dilution of the stock virus in tryptose phosphate broth. The embryos were incubated until death occurred and then refrigerated until the allantoic fluids were to be harvested. The infected fluid was pooled in a sterile flask, held at 4°C until checked for bacterial contamination. It was then portioned into sterile screw-cap vials of various sizes and stored frozen in the vials at -20°C until it was to be used. Harvests of up to two liters of pooled allantoic fluids were handled in this manner.

Tubes of frozen NDV (174HS) in allantoic fluid were removed from the

freezer and placed in the refrigerator for slow thawing at 4°C. Holding overnight under these conditions was adequate to ensure complete thawing. This procedure resulted in the formation of a flocculent precipitate which was centrifuged for 15 minutes at 2000 g in an International PR-2 refrigerated centrifuge. The supernatant was decanted by simply pouring off the liquid and gently shaking off the last clinging drop.

The precipitate button was taken up to the original volume by addition of sterile citrate-saline. The tubes were shaken vigorously to loosen the button and were immersed in a 37°C water bath until all the precipitate dissolved. The contents were pooled in a sterile flask and a sample was withdrawn from the pool for infectivity titer in egg embryos. This pooled material was the stock hemolytic virus for each experiment.

Erythrocytes

The red blood cells (rbc) used were obtained by cardiac puncture of white leghorn chickens. Blood was drawn via 18 gauge needle into approximately 1/10 of its volume of Alsever's solution in the syringe. The contents of the syringe were mixed by tilting gently and then gently expelled, with needle removed, into an erlynmeyer flask containing an equal volume of Alsever's solution.

The cells were centrifuged at 1200 g for 15 minutes. The supernatant was aspirated off and the cells were washed by resuspension in citrate-saline and recentrifugation at 1200 g for 15 minutes. The washing procedure was repeated three times. A stock dilution to approximately 10% rbc by volume of the packed cells was prepared using citrate-saline at the diluent.

A standard of erythrocyte concentration was defined so that the suspensions of red blood cells could be prepared reproducibly. The concentration of the red blood cell suspension was defined on the basis of the assigned value:

$$1\% \text{ rbc} = 6 \times 10^7 \text{ red blood cells/ml.}$$

The approximate 10% suspension was sampled by blood diluting pipette and diluted for convenient cell counts on a standard hemocytometer plate. The volume of additional dilution required to prepare a given final concentration of red blood cells was calculated on the basis of the actual cell count.

The addition of virus suspension to the erythrocyte suspensions will result in a reduced final red blood cell rbc concentration. This anticipated addition of virus suspension was deducted from the citrate saline diluent volume calculated above so that the final concentration of erythrocytes (after the addition of virus) would be a whole number. For example:

Let the original red blood cell concentration be 65×10^7 cells/ml;
let the protocol require the addition of 1 ml of virus suspension to each 4 ml of rbc suspension, yielding a final rbc concentration of 5.0% cells; and let a total of 150 ml of cells + virus be required.

$$5.0\% \text{ rbc} = 30 \times 10^7 \text{ rbc/ml}$$

$$v \times (65 \times 10^7) = 150 \times (30 \times 10^7)$$

$$v = 4500/65 = 69.2 \text{ ml}$$

$$150 \text{ ml (cells + virus)} = 120 \text{ ml cells} + 30 \text{ ml virus.}$$

Hence, 69.2 ml of the concentrated rbc suspension diluted to 120 ml with citrate saline will yield a final concentration after addition

of the virus suspension of exactly 5.0% rbc at the time of the mixing. Except where noted, fresh red blood cells were drawn for the commencement of each experiment. All of the cells collected were pooled prior to final dilution.

Calibration of Spectrophotometer Tubes

Cuvettes for the Spectronic 20¹ spectrophotometer were selected 13x100 mm test tubes. The tubes were filled with distilled water and matched to a control tube to within $\pm 0.5\%$ T. An index mark was scratched on each tube so that the necessary orientation in the cuvette holder could be reproduced.

Test tube cuvettes for the Coleman Jr.² spectrophotometer were prepared from 4 in. lengths of 7 mm o.d. standard wall pyrex tubing. A Coleman no. 6-106 12x75 mm round cuvette adapter was modified with a Teflon sleeve to accept the 7 mm cuvettes. The light slits of this holder were masked to reduce the incident beam to dimensions approximately equivalent to those of the Coleman no. 6-100 adapter. The 7 mm test tubes were matched by a procedure identical to that used above.

Cleaning and Decontamination

The spectrophotometer cuvettes containing virus and hemoglobin were drained into a stainless steel pan. The tubes were flushed with distilled water to remove all free hemoglobin, flushed with a disinfectant solution,

¹Bausch and Lomb, Rochester, New York 14602

²Coleman Instruments Corp., Maywood, Illinois 60154

and finally flushed with distilled water. All these rinsings were added to virus - hemoglobin drainings in the pan. The contents of the pan were decontaminated either by the addition of disinfectant or by autoclave sterilization before discarding. The decontaminated spectrophotometer tubes were washed thoroughly in Haemo-Sol¹ solution, flushed while inverted over running tap water, and finally rinsed over running distilled water. They were dried inverted in a 110°C oven.

Polyethylene filter cases contaminated with virus and hemoglobin were dropped into a pan of distilled water immediately after use. Immediately after the conclusion of the experiment, the cases were opened and the filters and support discs were removed and were autoclaved before disposal. The disassembled filter cases were immersed and agitated twice in distilled water rinses. The cases, now virtually free of hemoglobin traces, were decontaminated with disinfectant solution and finally rinsed with distilled water. All potentially infected rinsings were collected for autoclave sterilization before discarding. The filter cases were washed in Haemo-Sol solution, rinsed in running tap water, and finally rinsed in running distilled water. The cases were allowed to air-dry before reassembly.

Larger glassware, such as beakers, flasks and bleeding syringes, was rinsed, decontaminated, washed, and soaked overnight in chromic acid before final tap water and distilled water rinsing. All potentially infected drainings and rinsings from this equipment were decontaminated before discarding. The disposable plasticware exposed to virus was autoclaved before discarding.

¹American Hospital Supply, 2020 Ridge Avenue, Evanston, Illinois

EXPERIMENTAL

Commentary on Approach

A study of the kinetics of a chemical or biochemical process, in its essence, is an investigation of how the composition or state of a certain system changes with the passage of time. Kinetic information is a valuable tool to help elucidate the mechanism of the process being studied. In particular the mechanism of the hemolysis of erythrocytes by Newcastle disease virus has yet to be understood. The kinetics study to be developed here is an attempt to shed some light on the process of this hemolytic action.

It would be appropriate to begin by recalling the salient points from that which is known about NDV-induced hemolysis. The hemolytic activity of NDV has been considered to be enzymatic since the phenomenon was first reported [14, 69] and further developments have not brought conflicting evidence to light. Furthermore, this hemolysin seems to be firmly associated with the virus particle [32, 53, 69, 74]. On the basis of these observations it has been assumed for the purposes of this study that the virus particles may be treated in the first approximation as gross hemolytic enzymes with possibly more than one active site.

Some justification for this approach to the problem may be found in the fact that highly hemolytic preparations were routinely obtained without significant loss in infectivity titer by using the procedure for enhancing the hemolytic titer of the virus that was developed by Lucie [49]. The high infectivity titers accompanying these hemolytic preparations provide

an experimental basis for the assumption that the hemolytic units are intimately associated with the virus particles. Using this assumption to treat the virus suspensions as if they were solutions of a soluble enzyme, the infectivity titer was used as the ultimate criterion for equivalence between two preparations of hemolytic virus. A series of experiments was planned to show first the overall course of hemolysis induced by NDV, especially during the first few minutes after initiation, and then to measure the actual rate of hemolysis as the concentration of erythrocytes varied, keeping total hemolytic virus concentration constant. It was considered to be of prime importance to make all measurements of rate as close to "time zero" (the moment of mixing red blood cells and virus) as possible. The short-time approach proposed represents a departure from the usual procedure used in the study of hemolysis by NDV and perhaps some elaboration is appropriate.

Previously published studies on hemolysis by myxoviruses may be broadly categorized into two general classes, which may be of some significance to kinetics studies. For convenience, these classes are defined as follows:

Class I - The time of exposure of hemolytic myxovirus to red blood cells is held constant while other factors may be varied.

Class II - The time of exposure of hemolytic myxovirus to red blood cells is a controlled variable, while other factors are held constant.

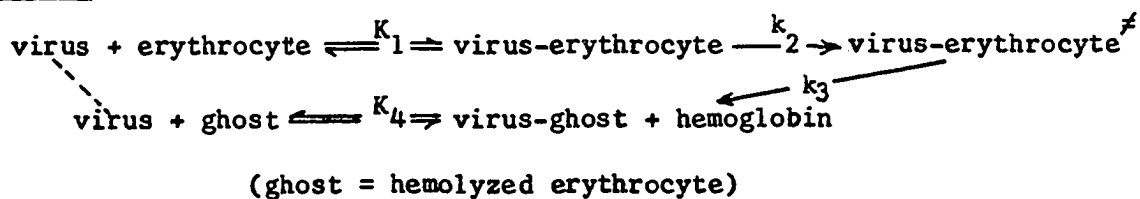
The studies of type Class I were often involved in the determination of hemolysin titer, or in the determination of the influence of various chemical and physical factors on the amount of hemolysis in the fixed

interval of time. These intervals were usually rather long, at least fifteen minutes and, in some cases, as long as fourteen to seventeen hours at 37°C [5, 17, 32, 34, 35, 49, 51, 52, 53, 54, 56, 62, 69, 70, 71, 73, 74, 75]. The studies of type Class II were involved in previous attempts to establish the kinetics of myxovirus hemolysis and in these cases some time intervals as short as approximately six minutes were involved [14, 36, 37, 69]. In this latter case, however, the rates of hemolysis observed were so rapid in comparison to the earliest time interval observed, at least fifty to sixty-seven percent of total hemolysis being completed at the time of the first experimental sampling [14, 36], that the calculation of the initial rate of hemolysis would be very unreliable by extrapolation to time zero from the given data. These initial hemolysis rate measurements take on increased importance in the light of the progressive reaction of hemolysis observed by Ponder and Cox [61] in the case of simple hemolysins and also demonstrated by Burnet [14] in the specific case of Newcastle disease virus hemolysis. These studies, in particular that by Burnet, suggest that the event wherein a red blood cell is altered by NDV so that hemolysis can take place is only a momentary incident in the overall time course of the appearance of free hemoglobin in solution. It is highly probable, therefore, that even with initial kinetics studies there would be some difficulty in determining which rate constants are actually being measured.

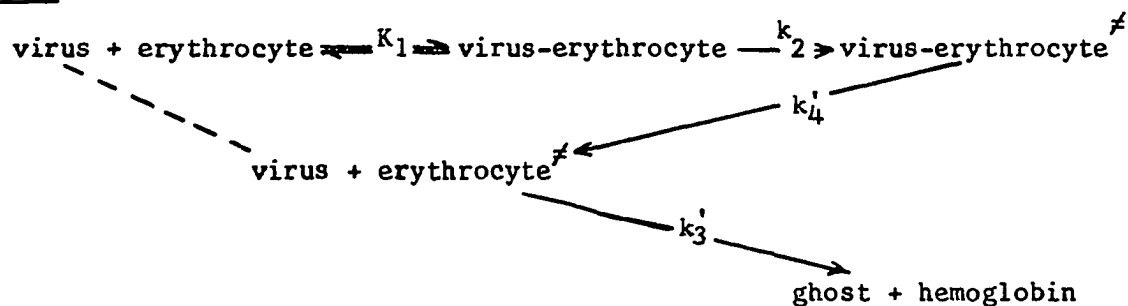
In biological systems it is very rare that the reactions are so simple that only one rate constant is involved between reactants and measurable products. Furthermore, it is not at all unusual for the apparent rate of

a reaction to change as the reaction proceeds - due to many factors, including the release of inhibitors or accelerators during the course of the reaction, side reactions between products and reactants, consumption of a critical intermediate or a reactant, etc. When it is possible to stop a sufficiently simple reaction at any chosen time after mixing, the rate of reaction can be measured as a function of the change in concentration of either reactants or products. If the side reactions, autoinhibition effects, etc., are unknown, however, this technique leads to ambiguity in determining which of several possible rates is being measured. The most direct approach to eliminate this problem is to measure the rate very shortly after mixing the reactants of interest. If the composition of the reacting materials is known at time zero (t_0), then at $t_0 + \Delta t$ the composition will not have changed much and there will be little opportunity for confusing side effects to occur if Δt is small compared to the total time necessary to carry the reaction to completion. In effect, the use of initial rate measurements allows the research worker to trade interpretative problems for technical problems. Consider the case of hemolysis by NDV. For the purpose of illustration the following hypothetical, alternative reactions (an elaboration on the pathways suggested by Sagik and Levine [69]) may be postulated:

Route 1



Route 2



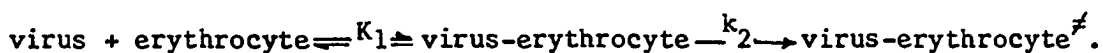
(ghost = hemolyzed erythrocyte)

where K_1 and K_4 are equilibrium constants; k_2 , k_3 , k'_3 and k'_4 are rate constants and erythrocyte \neq is a red blood cell which has been altered by the virus in such a way that loss of hemoglobin must follow. These two reaction routes are not necessarily mutually exclusive; but it is at least conceivable that hemolysis cannot commence until the virus particle has eluted (Route 2). This situation could be visualized if virus action is imagined as being like a punch which pierces a hole but plugs it by its own presence. Leakage of hemoglobin through the hole cannot begin until the virus "punch" is removed, i.e., until the virus has eluted as in Route 2. If the reaction of Route 1 is correct, the loss of hemoglobin is not prevented by the presence of the virus and in fact hemolysis may be far advanced before elution takes place. This type of mechanism could be visualized as a sort of "slashing" of the erythrocyte by the virus. The relative sizes of k_3 and K_4 would determine the degree of hemolysis suffered by the cell before the virus elutes.

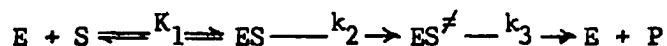
The form of the mechanisms proposed in Route 1 and Route 2 is similar to that of enzyme-substrate interactions and indeed the literature previously cited supports this view. Admittedly, the virus and the erythrocyte are each much more complex than the enzyme-substrate view implies,

but the gross mechanism of their interaction nevertheless responds to experimental manipulation very much like an enzyme-substrate system. The study of the kinetics of this interaction may therefore be pursued by methods applicable to enzyme-substrate systems.

From the enzyme kinetics point of view, only a portion of either Route 1 or Route 2 is of interest:



The reaction above is common to both Route 1 and Route 2 and may be considered the "hemolytic event" after which hemolysis is spontaneous; but it is incomplete for ordinary kinetics measurements as it stands. The completed reaction which is desired may be illustrated in the E and S notation of enzyme (E) - substrate (S) mechanisms:



where P represents a direct product of the action of the enzyme E on the substrate S. It is not possible to complete this mechanism for virus-erythrocyte interaction because no direct product of the virus action has ever been identified nor as yet can one differentiate between the attacked and unattacked red blood cells. Therefore, the usual methods of measuring enzyme kinetics, observing the rate of disappearance of substrate or the rate of appearance of product [64], cannot be applied. There are, however, some other methods by which the kinetics of the "hemolytic event" may be approximated.

The oversimplified hypotheses, Route 1 and Route 2, of the NDV-induced hemolysis process are not expected to reflect accurately the true mechanism. They are useful, however, to suggest kinetics experiments by

which the rate of the "hemolytic event" may be approximated and from which a more sophisticated hypothesis of the hemolytic action can be constructed. Two kinetics studies may be particularly appropriate:

- (1) the time-course of the cell attachment and elution of virus, and
- (2) the time-course of the appearance of free hemoglobin.

The first study would be most appropriate to a mechanism similar to Route 2 since elution takes place before hemoglobin is released. The second study is most significant for a Route 1 type of mechanism since free hemoglobin begins to appear before elution commences.

If comparable data can be obtained from these two approaches the correlation of both the proposed kinetics studies could be used to provide insight into the mechanism of hemolysis by NDV. The experimental details that follow are an attempt to develop the means to pursue these approaches to the hemolysis kinetics mechanism.

Exploratory Studies

To pursue the stated objectives, it will be necessary to penetrate previously unexplored aspects of NDV-induced hemolysis. It is logical to commence such an effort from a known point of reference by using or adapting previously reported techniques. Lucie [49] has shown that NDV-infected allantoic fluid can be separated into two portions of approximately equivalent infectivity titer. One portion, the precipitate, after reconstitution exhibits greatly enhanced hemolytic titer. The decantate has very little hemolytic activity and this difference in activity is characteristic of this treatment of the virus. The mechanics of this treatment

are well suited to routine use; thus, the method may be adopted as a technique for the preparation of hemolytic virus.

The technique which many authors used for hemolysis assay - that is, the incubation of hemolytic virus with red blood cells for an extended time (30 minutes at room temperature [48] to as much as 14 hours at 37°C [53]) before chilling the mixture, must be modified to observe the shorter intervals of interest to initial kinetics studies. The first few minutes of this reaction are potentially significant as can be seen in the paper by Hosaka [36] who, working with HVJ, obtained a hemolysis rate curve which shows hemolysis nearly 67% complete within ten minutes.

For the purpose of the preliminary trials, it will be sufficient to (1) show that the slow thawed infected allantoic fluid can be separated into two fractions which have markedly different hemolytic activity, (2) obtain some tentative information concerning the course of hemolysis during the first few minutes of reaction, and (3) evaluate the modified technique for performing this assay as described below.

Two 15 ml vials of frozen NDV were thawed and prepared for use as described in Materials and Methods. Fifteen ml of approximately 1% erythrocytes was placed in a large test tube and incubated at 37°C. The cells were mildly agitated with a twisted-ribbon glass stirrer blade rotated at 10 rpm. Pipettes used to remove aliquots were prewarmed to 37°C, as was also the virus suspension. Calibrated test tubes containing 2 ml of citrate-saline were chilled in an ice bath in preparation for their use to quench the reaction. A blank was prepared using 1 ml of the incubating rbc suspension + 3 ml of citrate-saline. Total hemolysis controls were not prepared for this experiment but such controls typically exhibit an

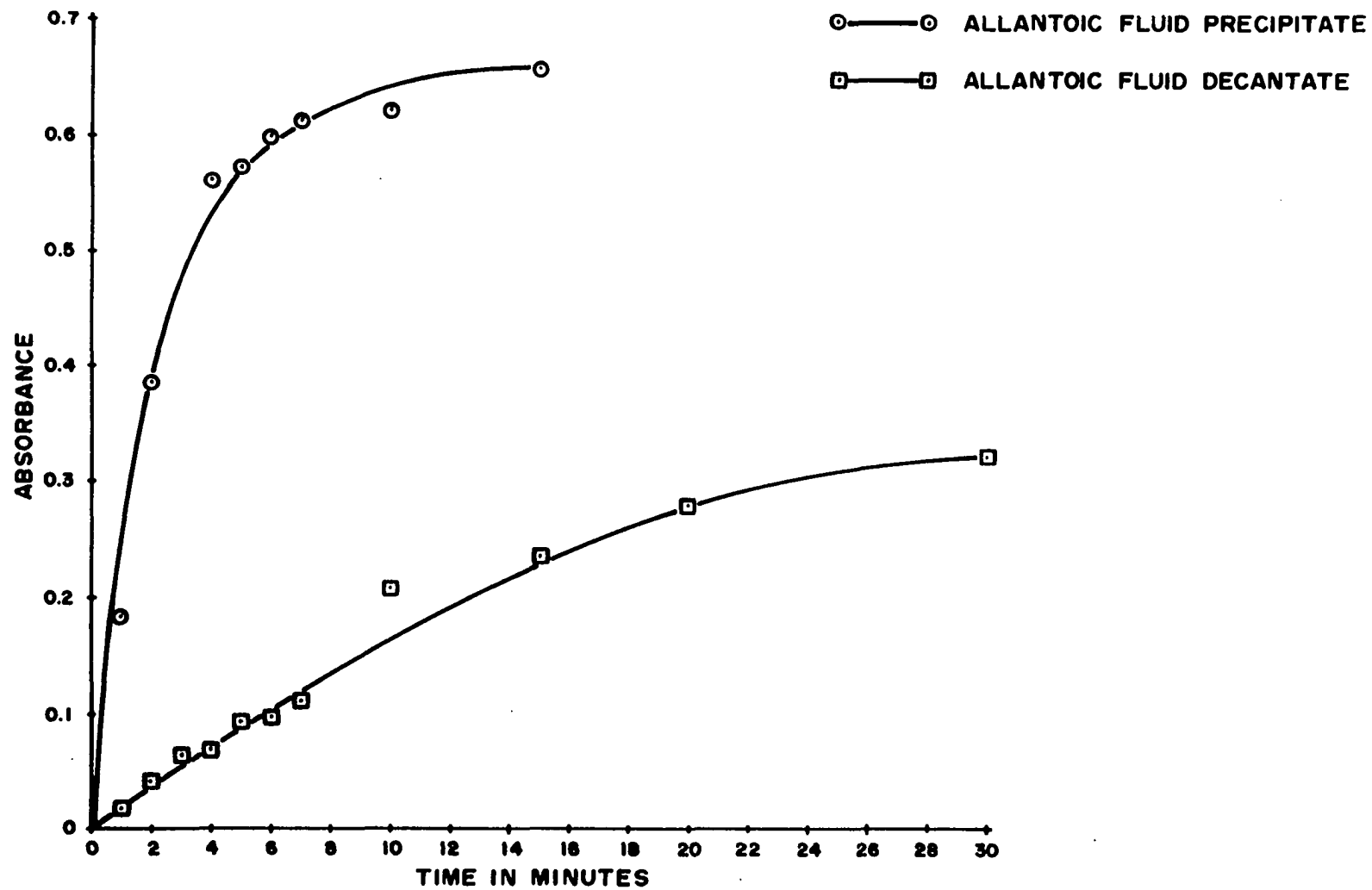
adsorbance of (approximately) 0.7 under the conditions of this experiment. At time zero, the moment of mixing of virus and rbc, 14 ml of the virus suspension from the allantoic fluid precipitate was added to the remaining 14 ml of 1% rbc. Two ml aliquots were removed from the stirring chamber and transferred to the tubes of cold buffer (quench) at the end of pre-determined intervals of time. The quenched aliquots were held in the ice bath until the end of the run. The tubes were centrifuged at 1800 g for five minutes and the absorbance of the supernatant was read in a Spectronic 20 spectrophotometer. The experiment was repeated using virus from the allantoic fluid supernatant. The results of both trials are shown in Figure 1 where the increase in absorbance is plotted against time.

Examination of Figure 1 reveals that (1) the sediment of slow thawed infected allantoic fluid contains a hemolytic agent much more potent than that residing in the supernatant, and (2) the rate of hemolysis by the re-constituted precipitate fraction is very rapid (more than 50% complete within the first two minutes). These observations indicate that the technique for the preparation of hemolytic virus will be adequate and is performing as expected, but the great speed of this reaction dictates that the assay technique be modified. This modification will be considered in the light of the requirements for the experimental approach to be introduced below.

Virus-Erythrocyte Attachment-Elution

The information obtained from the Exploratory Studies is sufficient to commence the investigation of the initial kinetics of NDV-induced

Figure 1. Comparison of hemolytic activity of NDV from two fractions of slow-thawed infected allantoic fluid. Infectivity titer of each fraction:
L. D.₅₀ = $10^{-8.9}$.



hemolysis. There were two approaches to this end mentioned at the conclusion of the Commentary on Approach. The literature previously cited indicates that the loss of hemoglobin by an erythrocyte is the final result of a chain of events initiated by the hemolysin. If the hemolysin is an enzyme, it cannot initiate this chain of events until it attaches or is attached to the substrate site of the erythrocyte. Furthermore, the hemolysin is no longer involved in the process of hemolysis after the attachment ceases, i.e., after elution. The kinetics of the hemolysin of NDV (the rate of the "hemolytic event") must be contained within this attachment-elution interval regardless of the rapidity of the appearance of liberated hemoglobin. Consequently "the time-course of the cell attachment and elution of virus" is expected to be the more direct of the two alternate approaches to the measurement of the rate of the "hemolytic event".

The paper of Sagik and Levine [69] is particularly pertinent to this approach. Their report of an attachment rate, elution conditions and multiplicity influences on virus-red blood cell interaction suggested the possibility that changes in the number of free infective units during the course of hemolysis might serve as a monitor of the progress of the reaction. The principle is that a short time after mixing virus and cells some of the virus becomes attached to the erythrocyte surfaces. If the cells are centrifuged and a sample of the supernatant is assayed for viral infectivity, it seems reasonable to expect a decrease in titer due to the virus attached to the sedimented cells. If sufficient time from the moment of mixing is allowed to elapse, however, the titer might be expected to rise again as the virus elutes (depending upon the homogeneity of the virus population).

This hypothesis forms the basis for the following experiments by which it is hoped to measure the duration of viral attachment to erythrocytes.

The infectivity assays of these experiments will involve the titration of NDV in egg embryos. This procedure requires that the virus inoculations be free of bacteria which could also kill the embryos. Sterile equipment and aseptic handling are necessary to maintain the inocula free of troublesome bacteria. To maintain sterility while removing samples of the incubating red blood cell-virus mixtures, the technique for hemolysis assay as used by Lucie [49] and others was adapted. The details are presented in the description of the experimental procedure.

Frozen allantoic fluid infected with NDV, strain 174HS, was thawed, centrifuged, and decanted in the usual way for preparation of hemolytic virus stock. The precipitate was taken up in sterile citrate-saline to which had been added 10% by volume of "Mix" as a precaution against contamination due to manipulative procedures. Aseptic techniques were used throughout the course of the experiment to minimize the possibility of bacterial contamination. The virus suspension was transferred to a sterile flask and dispensed with sterile pipets.

Chicken erythrocyte suspensions were prepared aseptically using sterile citrate-saline plus 10% by volume of "Mix". These were diluted to 1% cells ($\frac{1}{2}\%$ after addition of virus) and dispensed with a sterile Cornwall 2 ml pipettor fitted with the Cornwall dispensing tip. The flask containing the cell suspension was gently but thoroughly agitated during dispensing so that reasonably uniform aliquots could be prepared.

The undiluted virus suspension (designated "Initial Virus Concentra-

tion $\times 10^0$ ") was diluted serially in sterile 16x100 mm test tubes containing 1.8 ml of sterile citrate-saline-"Mix" as used above. These dilutions ($\times 10^{-1}$, $\times 10^{-2}$, $\times 10^{-3}$, etc.) were prepared in duplicate or in triplicate for each run. Controls were prepared using undiluted virus ($\times 10^0$), 1.8 ml of virus + 1.8 ml of sterile citrate-saline-"Mix".

The tubes of virus dilutions were incubated in the 37°C water bath as was also the flask of 1% rbc. When the two components had reached thermal equilibrium with the bath, 1.8 ml of 1% rbc was pipetted into the first tube of virus and the timer was started. At the end of the timed interval the tube was removed from the bath and chilled for 60 seconds in ice water while being vigorously swirled to accelerate heat transfer. The tube was immediately transferred to the centrifuge and the cells sedimented at 800 g. The decantate was immediately removed with a sterile Pasteur pipet, transferred to another sterile tube and reserved for the infectivity assay. This procedure was repeated throughout the course of the experiment where times of less than 60 seconds of hemolytic interaction were involved. When the interaction time was 60 seconds or more, the rbc were added to the duplicate or triplicate sets in sequence and the tubes were removed from the bath in the same sequence so that variations in timing could be minimized.

Virus assay was done by titration in chick embryos using LD₅₀ end-point calculations by the method of Reed and Muench [63]. Death by NDV was confirmed by hemagglutination test; only positive tests were counted as deaths. The raw data reflect the effect of both serial dilution and any loss in titer due to virus-rbc attachment. Table 1 shows the infectiv-

Table 1. Influence of hemolytic conditions on infectivity titer

Dilutions	Uncorrected titer at time:									Corrected titer at time:								
	0"	10"	30" ^a	30" ^b	30" ^c	60"	90"	120"	180"	0"	10"	30" ^a	30" ^b	30" ^c	60"	90"	120"	180"
Con- trol	8.33 8.00	8.23 8.50	8.50 8.75	8.50 8.00	4.16 4.50	8.00 8.67	8.50* 8.23* 8.33	8.00 7.75	8.45 8.77	8.33 8.00	8.23 8.50	8.50 8.75	8.50 8.00	8.16 8.50	8.00 8.67	8.50* 8.23* 8.33	8.00 7.75	8.45 8.77
10 ⁰	8.5 8.5	8.5 8.0	7.0 8.0	7.5 7.5	- -	8.5 8.0	7.5 7.5	8.67 8.33	8.75 9.00	8.5 8.5	8.5 8.0	7.0 8.0	7.5 7.5	- -	8.5 8.0	7.5 7.5	8.67 8.33	8.75 9.00
10 ⁻¹	7.0 7.5	6.5 6.5	7.0 7.5	8.0 7.0	- -	7.5 7.0	6.5 7.0	7.23 7.25	6.67 7.00	8.0 8.5	7.5 7.5	8.0 8.5	9.0 8.0	- -	8.5 8.0	7.5 8.0	8.23 8.25	7.67 8.00
10 ⁻²	5.5 6.0	6.0 6.0	5.5 5.0	6.5 5.5	- -	5.5 5.0	6.0 5.5	5.67 5.50	5.50 5.50	7.5 8.0	8.0 8.0	7.5 7.0	8.5 7.5	- -	7.5 7.0	8.0 7.5	7.67 7.50	7.50 7.50
10 ⁻³	5.5 4.5	5.0 5.5	4.5 5.0	5.0 4.5	- -	5.5 4.0	4.5 4.5	5.33 5.00	5.00 4.68	8.5 7.5	8.0 8.5	7.5 8.0	8.0 7.5	- -	8.5 7.0	7.5 7.5	8.33 8.00	8.00 7.68
10 ⁻⁴	3.0	3.0	3.5	4.0	4.33 4.67	4.0	3.5	-	-	7.0	7.0	7.5	8.0	8.33 8.67	8.0	7.5	-	-
	3.5	3.5	3.0	4.5	4.67	< 2	3.5	-	-	7.5	7.5	7.0	8.5	8.67	< 6	7.5	-	-
10 ⁻⁵	3.5 2.0	1.5 3.0	< 0.5 < 0.5	3.0 2.0	2.50 3.00 3.33	2.0 < 1	2.5 2.0	- -	- -	8.5 7.0	6.5 8.0	< 5.5 < 5.5	8.0 7.0	7.50 8.00 8.33	7.0 < 6	7.5 7.0	- -	- -
10 ⁻⁶					2.00 2.67 2.67	1.5								8.00 8.67 8.67	7.5			

^aVirus + 1% fresh rbc (i.e., as usual)

^bVirus + 1% 48-hour-old rbc - to observe age effect

^cRecheck of anomalous 10⁻⁵ point of 30"^a; fresh rbc

* See Text

ity titer observed for each exposure time and the infectivity titer corrected to account for serial dilution. Data reported to two significant figures were obtained using two embryos per titration dilution; three significant figures are reported where four embryos per titration dilution were used. The blanks marked were not treated with antibiotics so that any significant effect of the antibiotic on the infectivity titer might be observed.

The data of Table 1 show that no significant decline in infectivity titer was observed under the experimental conditions described. There are variations in the observed titer, especially where only two eggs were used per dilution. The pattern of this variation appears to be quite random, however, and is not far from what might be expected after repeated handling of a Newcastle sample even if not exposed to erythrocytes. It is significant to note in this regard that the virus-to-rbc ratios in this study varied from (in round numbers) 10:1 to 1:1000.

The previous experiment showed that hemolysis proceeds to a significant degree within the experimental conditions of this last study. The hemoglobin released was not measured spectrophotometrically but hemolysis was noticeable as a pink coloration in all supernatants except those at the earliest time intervals. A rationale concerning the failure to observe the expected decrease in infectivity titer is limited by the knowledge that hemolysis has nevertheless occurred. One possibility is that the virus attached and eluted from the red cells so rapidly that even the zero time experiment was too cumbersome to reveal the decrease in titer for such a short interval. It is also conceivable that there is little or

no identity between infective and hemolytic particles. This possibility is not very attractive but the variance of infectivity assay data does not allow it to be ruled out on the basis of the information available. In either case a major revision in experimental design would be required to attempt to examine these possibilities in detail. Such an effort, although most worthwhile, would be a digression from the primary motive of the research. Further work to estimate the initial kinetics of NDV-induced hemolysis will therefore be based upon "the time-course of the appearance of free hemoglobin" (the second kinetics study alternative proposed in the Commentary on Approach).

Time-Course of the Appearance of Free Hemoglobin

The use of liberated hemoglobin as a monitor of the rate of the "hemolytic event" has been proposed as an alternative to the approach taken in the previous section. This method has been used previously, although not for initial kinetics, by other workers who have studied the kinetics of viral hemolysis [14, 36]. This approach has merit even for initial kinetics since the appearance of free hemoglobin is positive evidence that the "hemolytic event" has occurred. The primary disadvantage to the use of this method for initial rate measurements is that the liberation of hemoglobin is probably somewhat more remote from the "hemolytic event" than in the approach of the previous section. This is not a serious problem and needs only to be recognized in the interpretation of the data.

Rapid sampling technique - syringe pipet

In the Exploratory Studies it was observed that more rapid sampling

would be required than the pipetting technique would permit. The modification of the technique used in the previous section was chosen to accommodate the added requirements of bacterial sterility. A sterile system is no longer necessary for the measurement of liberated hemoglobin. The relaxation of this constraint provides an opportunity to further improve the sampling procedure so that uniform samples may be drawn and thermally quenched as quickly as possible.

There are two possible approaches to more rapid sampling: a faster means for removing samples at progressively timed intervals from a stock mixture of red blood cells and virus, or to mix virus with rbc in small, single-sample quantities which could be removed from incubation and chilled at timed intervals. The latter method has the advantage that samples could be taken as rapidly as desired by simply removing the incubating tube from the water bath and plunging it into an ice bath. This method, however, has two serious uncertainties; the efficiency of mixing virus with the rbc suspension in small tubes during addition and incubation, and the added difficulty of chilling both the warm test tube and its contents quickly to quench the reaction. These difficulties are overcome by using a larger pool of erythrocytes since it is possible to introduce constant stirring and to quench the samples in separate chilled tubes. These advantages, however, are not gained without some sacrifice. Controlling the sample time from the larger pool is inherently more difficult than with the smaller tubes. The use of prewarmed pipettes as in the preliminary experiments has an effective limiting speed of about one sample per minute, which is imposed by the slow draining time of the pipettes. It will be

necessary to remove samples quantitatively in order to obtain data which could be interpreted in terms of rate equations. Therefore, an improved sampling technique from the larger pool would require both a more rapid uptake and transfer of the sample as well as an assurance of a reproducible and quantitatively fixed amount of material with each sample removed. A hypodermic syringe properly adapted to deliver a fixed volume is a very likely candidate for this type of sampling procedure.

To meet this need for very quick, reproducible sampling of the virus-rbc mixture, the apparatus shown in Figures 2 and 3 was designed. Figure 2 shows a modified B-D Cornwall syringe and metal pipetting holder¹. The modification, removal of the spring and substitution of a friction-fit rubber septum, is intended to allow the syringe to remain closed until such time as the operator raises the plunger with the thumb-ring adapter. This modification restricts the contents of the apparatus to the material which will be retained in the needle and hub assembly when the plunger is closed. With the plunger set for a 2 ml delivery, the contents of the needle and hub assembly would be essentially negligible in their contribution to the sample volume. Figure 3 shows the modified Erlenmeyer flask with a syringe port positioned in the side to allow the needle to enter close to the bottom of the flask. The entry port was set at a convenient angle so that the syringe could be inserted and withdrawn quickly for rapid sampling. The flask was clamped in a 37°C water bath; immersion was adjusted to provide an adequate zone of incubation without risk of flooding the

¹Becton, Dickinson and Co., Rutherford, New Jersey

Figure 2. Modified Cornwall 5 cc syringe pipettor and cannula.

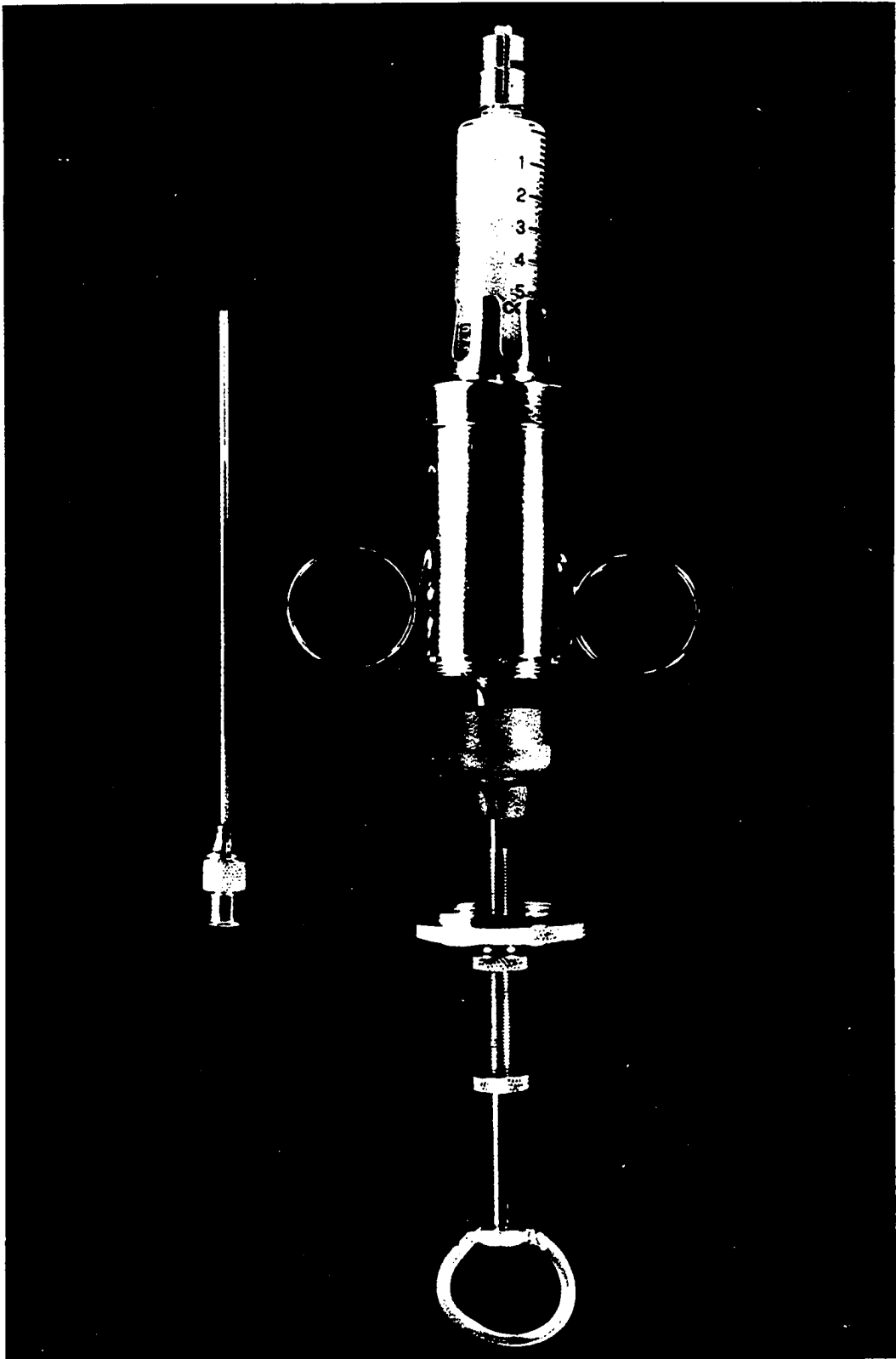
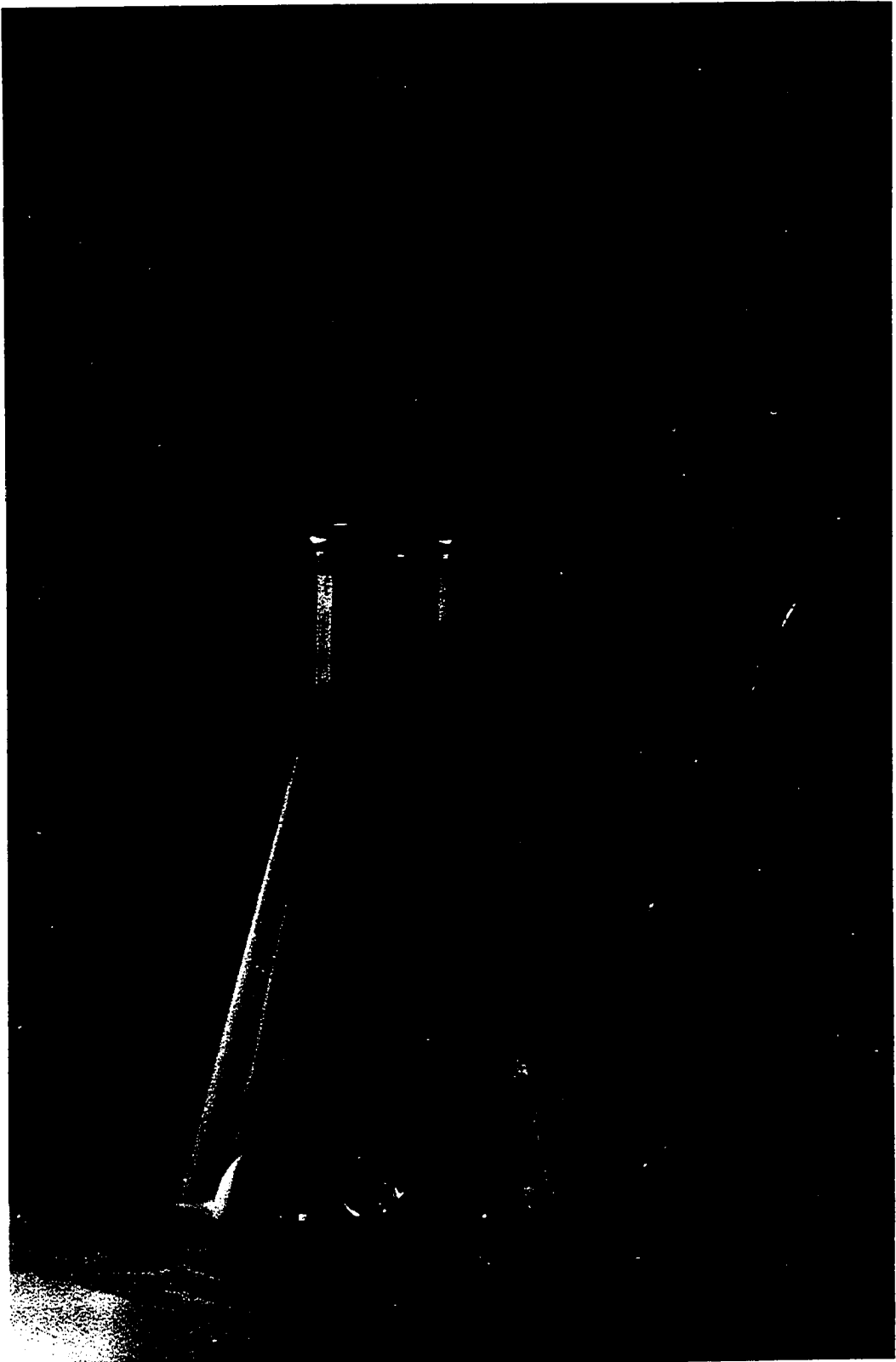


Figure 3. Modified Erlynmeyer flask and Teflon stirrer.



side arm opening. A Teflon paddle for the half-moon stirrer was cut from 3/32" stock. Several 3/16" holes were drilled through the paddle to increase stirring turbulence at low rotational speeds.

The mechanical operation of the sampler proceeded as follows:

- (1) Substrate erythrocyte suspension dispensed into sidearm flask (Figure 3).
- (2) Flask partially immersed in 37°C water bath.
- (3) Slow mechanical stirring disperses cells as temperature comes to 37°C.
- (4) Blank aliquot of cells removed from the flask.
- (5) Closed dispenser syringe (Figure 2) inserted into sidearm.
- (6) Hemolytic virus at 37°C added to remaining cell suspension and time clock started.
- (7) Approximately 8 seconds before time t , syringe filled and emptied twice in succession to pre-warm the syringe. Third fill is removed and dispensed into quench at time t .
- (8) Closed syringe replaced in sidearm and above sampling procedure repeated for each selected time interval.

It was found that by this technique aliquots could be removed at intervals as short as 10 seconds. This represents a distinct improvement over the technique used in the previous experiments and may prove to be a satisfactory method for sampling provided that the technique itself does not contribute appreciably to hemolysis.

The mechanical stirring used in this apparatus (Figure 3) is more vigorous than that of the previous experiments. Vigorous agitation of

the erythrocyte suspension is desirable to disperse the cells uniformly and to ensure rapid mixing of the added virus suspension at t_0 . Erythrocytes are subject to mechanical damage, however, and too vigorous agitation may cause considerable hemolysis in the blanks. It is advisable, therefore, to determine how much mechanical stirring contributes to the hemolysis of the blank.

The hemolytic effect of stirring was studied using 0.5% rbc as the substrate. Fifty milliliters of the cell suspension was placed in the modified flask shown in Figure 3. The flask was partially immersed in the 37°C water bath and agitated gently by hand until the erythrocyte suspension came to bath temperature. A "zero time" aliquot was drawn in the manner described for the syringe sampling technique. The mechanical stirrer was then started and additional samples were withdrawn at later times as shown in Table 2. No quench was used so that the dilution effect

Table 2. Hemolysis of erythrocytes induced by stirring

Time	Absorbance	Time	Absorbance
0 min	0.00	15 min	0.01
1 min	0.00	20 min	0.00
5 min	0.00	30 min	0.00
10 min	0.00	50 min	0.00
		60 min	0.01

Substrate: 0.5% erythrocytes in citrate-saline

No virus

Spectrophotometer: Spectronic 20

on the absorbance could be avoided. The "zero time" blank was used to set the spectrophotometer to zero absorbance and all subsequent aliquots were compared to this. The results indicate that mechanical hemolysis is negligible in this system and that no correction need be applied to the observed absorbance for periods less than one hour.

A qualitative extension of the Exploratory Studies was undertaken to test the utility of the syringe pipetting technique and to gain some additional information about the early stages of NDV-induced hemolysis. The new technique was applied to a hemolysis rate experiment in which virus at three concentration levels was added to a fixed amount of red blood cells. For enzyme-moderated reactions at substrate saturation, the rate of conversion of substrate to product is proportional to enzyme concentration. From this study it was hoped to learn:

- (1) The actual utility of the syringe pipetting technique.
- (2) More detail of the first few minutes of hemolysis.
- (3) An indication of the relationship between virus concentration and hemolysis rate under the experimental conditions employed.

Infected allantoic fluid precipitate was reconstituted in citrate-saline as usual. Two portions of this material were diluted further to give three final preparations: undiluted virus, 1:5 (undiluted virus: final volume) dilution, and 1:10 dilution. These dilutions were prewarmed to 37°C before addition to the erythrocyte substrate.

Forty-five ml of 1% erythrocytes was incubated at 37°C in the side-arm flask (Figure 3). Mild agitation with the Teflon paddle stirrer kept the cells in suspension. Five ml of the virus dilution selected for the

run was added by pre-warmed serological pipet at time zero. The syringe pipet, set for a 2.0 ml delivery, was immediately pumped twice and the third fill was transferred to the quench at the end of the specified time interval. Blanks were prepared from the 1% rbc stock. Total hemolysis was achieved by adding a small amount of saponin to the appropriate dilution of 1% rbc.

Quenching was accomplished as in the preliminary experiments, using 2.0 ml of citrate-saline in test tube receivers chilled in an ice bath. At the end of the experiment the tubes were centrifuged at 800 g and the percent transmittance of the supernatant was read in the Spectronic 20.

The results of the experiment are tabulated in Table 3, and Figure 4 is a plot of the data in the form of percent hemolysis versus time. Percent hemolysis is defined as:

$$\% \text{ hemolysis} = \frac{\text{absorbance sample} \times 100}{\text{absorbance total hemolysis}} .$$

The experimental results just presented indicate that the syringe pipetting technique is adequate to obtain aliquots at intervals of about ten seconds. The curves of Figure 4 behave more-or-less as expected although there is some ambiguity in their interpretation. The rate of hemolysis by 1:5 diluted virus is approximately twice that of 1:10 diluted, but the rate of undiluted virus falls far short of 5-times the rate of 1:5 diluted virus. This observation could be due to one or more of the following conditions:

- (1) Inadequate supply of substrate (erythrocytes) for maximum hemolysis rate by undiluted virus.

Table 3. Absorbance vs. time data for three concentrations of 174HS

45 ml 1% rbc + 5 ml undiluted 174HS				45 ml 1% rbc + 5 ml 1:5 174HS				45 ml 1% rbc + 5 ml 1:10 174HS			
Time (sec)	% T ^a	A ^b	% Hem ^c	Time (sec)	% T ^a	A ^b	% Hem ^c	Time (sec)	% T ^a	A ^b	% Hem ^c
10	97.0	0.009	0.98	15	96.3	0.012	1.33	10	95.5	0.016	1.74
20	79.0	0.098	10.64	25	89.5	0.044	4.78	20	96.0	0.014	1.52
30	59.8	0.220	23.9	39	82.0	0.082	8.91	30	95.5	0.016	1.74
40	45.9	0.334	36.3	48	78.5	0.101	11.0	40	91.5	0.035	3.80
50	40.0	0.394	42.8	56	74.8	0.122	13.2	50	89.2	0.046	5.00
60	37.1	0.427	46.3	65	67.8	0.165	17.9	60	86.3	0.060	6.52
90	31.2	0.502	54.5	95	62.8	0.198	21.5	90	82.0	0.082	8.91
120	28.5	0.542	58.8	126	57.7	0.235	25.5	120	79.9	0.093	10.1
150	25.5	0.589	63.9	150	55.9	0.249	27.0	150	77.0	0.110	11.9
180	23.7	0.621	67.4	180	57.5	0.246	26.7	180	75.0	0.121	13.1

Blank I = 100% T = 0.000 A

Blank II = 88.5% T (vs. Blank I) = 0.007 A

Average Blank = 0.004 A

Saponin I = 12.5% T (vs. Blank I) = 0.903 A

Saponin II = 11.5% T (vs. Blank I) = 0.939 A

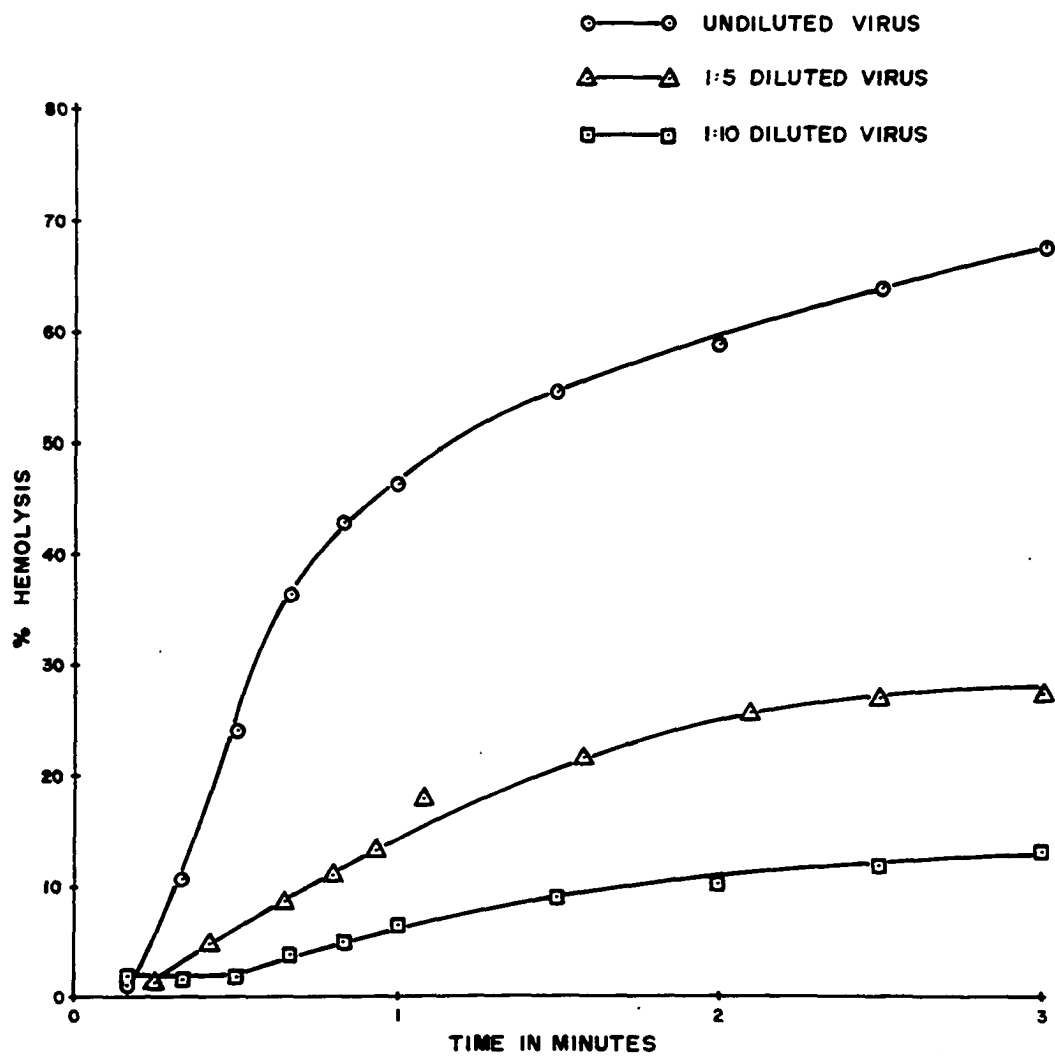
Average saponin = 0.921 A

^a100% T = Blank I

^bA = -log T - 0.004

^c% Hem = A/0.921

Figure 4. Percent hemolysis versus time for three concentrations of hemolytic NDV.



(2) Clumping or association of hemolytic units in undiluted virus.

(3) Shortcomings in the assumptions:

virus = enzyme

erythrocyte = substrate

free hemoglobin = product.

Furthermore, all of these curves must extrapolate to the origin (0.0% hemolysis at $t = 0$) but they clearly do not do so without creating an inflection in the curve. It seems highly unlikely that the inflection, if real, occurs in the vicinity of the first data point for each concentration of virus, as it appears in Figure 4. Further efforts to characterize this hemolytic action may shed light on this peculiarity.

The technique for rapid sampling seems to work very well, thus enabling a further exploration into the early kinetics of hemolysis. The previous experiment was unable to distinguish between three alternatives for the non-proportional effect of virus dilution on hemolytic rate. One of these alternatives was that 0.9% (final concentration after addition of virus) erythrocytes is insufficient to provide substrate saturation for the virus. The following experiment was undertaken to evaluate the effect of increased erythrocyte concentration on the hemolysis rate.

Effect of erythrocyte concentration on hemolysis rate

To determine the concentration of substrate (erythrocytes) necessary to allow maximal hemolysis by a fixed concentration of virus, a standard amount of hemolytic NDV will be added to portions of a graded series of rbc suspensions. The hemolysis is to be quenched 60 seconds after mixing and the cells will be sedimented by centrifugation. The absorbance of the supernatant solutions will be read and the absorbance plotted versus

the rbc concentration.

This experiment will be carried out in two stages:

- (1) A qualitative, wide-range assay to determine an approximate level of substrate concentration necessary to provide saturating conditions and
- (2) A quantitative assay using only a few concentrations to obtain the virus:rbc saturation ratio and an estimation of the maximum hemolysis rate.

The first experiment, being qualitative and involving many dilutions of red blood cells, will best be handled by mixing rbc and virus in separate test tubes and adding cold citrate-saline quench to these tubes. The technique is similar to that used in the "reduction of infectivity titer" experiments except that aseptic handling is not required.

For the first attempt, to establish the general level of substrate saturation, the stock suspension of hemolytic NDV, strain 174HS, was prepared in the usual way but not titered in egg embryos. Preparation of the hemolytic stock routinely yields infectivity titers in the vicinity of $10^{-8.5}$; hence dilution by volume would be qualitatively reproducible. The stock was diluted further by a factor of 20 for use in the experiment. Red blood cell suspensions of 1%, 2%, 3%, 4%, 5%, 10%, and 15% cells (dilution by volume) were prepared from freshly harvested chicken erythrocytes. One milliliter of the diluted virus suspension was pipetted into each of 21 13x100mm test tubes matched for use in the Spectronic 20. The rbc suspensions and the tubes of virus were incubated separately at 37°C so that there would be negligible change in temperature upon mixing. One milliliter of the selected rbc suspension was pipetted into each of three

of the tubes of virus and this triplicate inoculation was repeated for each suspension of erythrocytes. The mixtures were incubated for 60 seconds after mixing. At the end of the time interval, two milliliters of 0°C citrate-saline quench was added to each of the tubes which were then plunged into ice water. The tubes were immediately centrifuged at 800 g and put aside for spectrophotometric assay. Blanks were prepared using 1 ml of each rbc suspension plus 3 ml citrate-saline in the matched 13x100 mm test tubes. Total hemolysis controls were prepared similar to the blanks, with the addition of a small amount of dry saponin. Spectrophotometric assay was carried out in the Spectronic 20, assigning the blank prepared from 1% rbc the reference value 0.000 absorbance.

The results of this experiment are shown in Table 4. It is immediately apparent that the data are erratic. The cause of this could be attributed to several factors: poor preparation of blanks (absorbance of blanks too high), poor preparation of controls (hemolysis not really complete), nonuniform distribution of virus and/or red blood cells in the tubes, or other factors. The dilution factor, 1:20, used to prepare the virus suspension for this experiment makes it unlikely that nonuniform distribution of virus is a significant factor. Steps were taken to minimize the other sources of error and the experiment was repeated.

The red blood cell suspensions were prepared as before using greater care to minimize hemolysis due to the mechanical shock of handling. The incubated suspensions were thoroughly agitated until all traces of sedimented cells were removed from the bottom of the flask before pipetting the rbc into the tubes for blanks, controls, and viral hemolysis. Controls

Table 4. Absorbance versus red blood cell concentration (60-sec hemolysis by NDV strain 174HS)

% cells	Blank absorbance	Absorbance ₆₀			Average A ₆₀	Net* absorbance	Control (Saponin)	% hemolysis
		Triplicate data						
1	0.000A	0.040A	0.045A	0.038A	0.041A	0.041A	0.550A	7.5%
2	0.028	0.026	0.024	0.038	0.029	0.001	1.2	< 1.0%
3	0.028	0.043	0.037	0.034	0.038	0.010	1.5	< 1.0%
4	0.045	0.071	0.071	0.075	0.072	0.027	2.0	1.3%
5	0.056	0.067	0.075	0.068	0.070	0.014	> 2	-
10	0.067	0.152	0.144	0.163	0.153	0.086	> 2	-
15	0.130	0.210	0.210	0.240	0.220	0.090	> 2	-

*Net A = Average A₆₀ - Blank A

(saponin treated for total hemolysis) of absorbance greater than 1.0 were diluted 1:5 with distilled H_2O to assay the absorbance. The value obtained for the diluted control was multiplied by 5 to obtain the absorbance of the undiluted material. The triplicate viral hemolysis assay was quenched and centrifuged as before. The tubes were racked and collected for spectrophotometric assay at the conclusion of the experiment. The results of this 60 second hemolysis experiment are presented in Table 5.

Table 5. Absorbance versus red blood cell concentration^a

% cells	Absorbance blank	Absorbance ₆₀ triplicate data			Control (Saponin)
1	0.000	0.031	0.070	0.058	0.590
2	0.008	0.043	0.133	0.120	1.25
3	0.009	0.045	0.110	0.085	0.925
4	0.020	0.053	0.145	0.135	1.41
5	0.020	0.110	0.285	0.235	3.30
10	0.036	0.153	0.445	0.355	6.25
15	0.065	0.175	0.520	0.420	8.75

^a60 second hemolysis by NDV, Strain 174HS.

The data from this repeat of the attempt to establish the level of erythrocyte concentration required to obtain maximal hemolytic rate are in even poorer agreement than before. The differences among the tubes of each triplicate were so great that they were visible to the naked eye.

The key to the difficulty was found as the tubes were being polished for insertion in the spectrophotometer. The button of cells packed in

the bottom of each tube was leaking hemoglobin into the supernatant. The absorbance was changing moment by moment as this leakage continued. The phenomenon was not noticeable in the tubes containing only a few cells but leakage in the 5%, 10% and 15% tubes was quite apparent. By inference from the data, this leakage must also be taking place in the tubes of lower concentration as well.

A few short experiments were carried out to determine whether it was possible to get satisfactory supernatants with the fastest possible handling and centrifugation. The virus prepared for the previous experiment was mixed with 10% rbc (1:1 virus/rbc suspension) at 37°C. After 60 seconds the incubating tubes were removed from the water bath, quenched and plunged into an ice bath for a few seconds. The chilled suspension was transferred immediately to the centrifuge and spun for 3 minutes at 800 g. As soon as rotation stopped the tubes were examined for hemoglobin leakage from the sedimented cells. Leakage was evident in every trial. Centrifugation time was reduced to less than two minutes total time and the experiment was repeated. Again leakage of hemoglobin was apparent. These observations shed serious doubt on the value of thermal quenching and centrifugation as procedural steps in the study of hemolysis in its early stages.

The above information provides new inferences concerning some of the data already presented. Hemolytic "drift" may account for the apparent distortion near the origin of the curves presented in Figure 4. This is particularly true of that experiment since the "quenched" tubes of virus were allowed to stand in the ice bath until the end of each trial. Even those aliquots quenched at 10 seconds had more than 3 minutes to continue

hemolysis while subsequent aliquots were removed and quenched.

These inferences have little application, however, to the studies previously published by other workers. Their virus-erythrocyte incubation times were so long by comparison that the further change after quenching was negligible. This advantage must be sacrificed for the study of initial kinetics by measurement of the rate of appearance of free hemoglobin. A technique must be developed which will be more suitable than quench-centrifugation for initial hemolysis rate measurements.

Modified rapid sampling technique - syringe filter

At present it is necessary to study the initial kinetics of hemolysis by spectrophotometric measurement of hemoglobin liberated during a known interval. This procedure requires a solution free of cellular debris and other turbidity, the removal of which takes time. On the basis of the information gained above, there are three alternative approaches to a more reproducible measurement of hemolysis rate using hemoglobin assay:

- (1) Conduct the assay in the presence of the turbidity.
- (2) Stop the hemolysis completely at a chosen time, thus providing all the time needed for removal of turbidity.
- (3) Find a means to remove the turbidity more rapidly than present methods allow.

Neither of the first two alternatives are likely to prove feasible in this system. Turbidity corrections for absorbancy measurement are particularly complicated in this system where erythrocytes are likely to undergo shape and volume changes during hemolysis [59, 60]. The mechanism of hemoglobin

loss by a hemolyzing erythrocyte is too poorly understood at present to permit the actual implementation of alternative (2). There is, however, some hope of finding a practical, rapid method to remove the hemolyzing red blood cell from the solution. There are several techniques which are used to remove large particles from suspension: dialysis, centrifugation, flotation, flocculation, and filtration. All of these are usually slow processes except for filtration. Filtration would appear to hold the greatest promise for speed and utility.

There are several potential problem areas which must be considered during the evaluation of a filtration method for this system:

(1) Erythrocytes by their nature will be difficult to filter. They are a tissue cell and will easily pack in layers on the surface of a filter.

(2) The effect of pressure on hemolyzing red blood cells is unknown but is probably a strong influence on the rate of hemolysis. Pressure on the filtered erythrocytes would be involved in either vacuum or pressure filtration. A dense layer of red blood cells on the surface of the filter would further increase the pressure differential.

(3) Most samples for filtration would contain high titers of viable virus. A filtration system capable of removing red blood cells must also be compatible with the precaution and procedure of routine handling of infectious materials.

The considerations just outlined provide the basis for the following criteria for a filtration method:

- (1) It must be efficient, removing all erythrocytes.
- (2) It must not contribute significantly to hemolysis.
- (3) It must allow control of the infectious particles.

In addition;

- (4) It must be fast and reproducible.
- (5) It must permit accurate timing for kinetics measurements.
- (6) It must be economical in the consumption of both erythrocytes and virus.

There are several standard syringe filter systems commercially available that could provide the speed and utility required in (4), (5), and (6). The syringe pipetting technique has already demonstrated the utility of a hypodermic syringe for rapid sampling. The real problem remaining is to find a filter satisfying (1), (2), and (3).

Filter design The Swinny Hypodermic Adapter and the Tenite disposable filter cases offered by Millipore Filter Corporation¹ and the Gelman Cytosieve² filter cases were tried using Gelman membrane filters, Millipore filters, and Millipore glass fiber pre-filters. Both the Gelman membrane filters and the Millipore filters plugged instantly when an attempt was made to filter a red blood cell suspension. Only the Millipore glass fiber pre-filter^{1,3} succeeded in removing the cells. This success is probably due to a slow penetration of the filter by the

¹Millipore Filter Corp., Bedford, Massachusetts

²Gelman Instrument Co., 600 S. Wagner Rd., Ann Arbor, Mich. 48106

³Millipore No. AP2502500, Thick

cells, which prevents them from packing on the surface and plugging the filter.

The performance of the Millipore pre-filter material was remarkable. At least 3 ml of 7% rbc (1.26×10^9 cells) could be rendered cell-free in less than 5 seconds with almost no discernable back-pressure. The thickness of the fiber pad, however, prevented the Swinny Adapter from achieving a leak-proof seal. The Millipore disposable Tenite filter cases worked well mechanically with the filters but had a large dead-space above the filter. The Gelman Cytosieve case in combination with the Millipore pre-filters was found to be a nearly ideal combination. With this combination it was possible to set up more than 100 filters at the beginning of an experiment and they were easily disassembled after use for washing and decontamination.

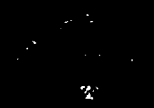
The bottom of the Cytosieve filter case was fitted with a male Luer hypodermic needle (Becton, Dickinson adapter for VacuTainer to standard Luer needle)¹ to facilitate transfer of the filtrate to small spectrophotometer tubes. A cytosieve support disc² was placed in the recess of the filter case and a Millipore glass fiber pre-filter was placed on top of the support disc. The disassembled filter is shown in Figure 5. The filter case was closed with the cover and the assembly was clamped together with a No. 18 ball joint clamp. A pre-filled syringe was connected to the cover of the assembled filter and the contents of the syringe were expelled through the filter into the spectrophotometer tube. The con-

¹ Becton, Dickinson and Co., Rutherford, New Jersey


² Gelman Instrument Co., 600 E. Wagner Rd., Ann Arbor, Mich. 48106

Figure 5. Disassembled view of syringe filter as used to remove red blood cells from suspension.

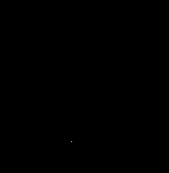
DISASSEMBLED FILTER



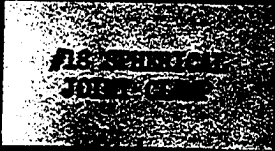
CYTOSIEVE FILTER
CASE COVER




MILLIPORE GLASS FIBER
PRE-FILTER



CYTOSIEVE FILTER
SUPPORT DISC



CYTOSIEVE FILTER
CASE BOTTOM



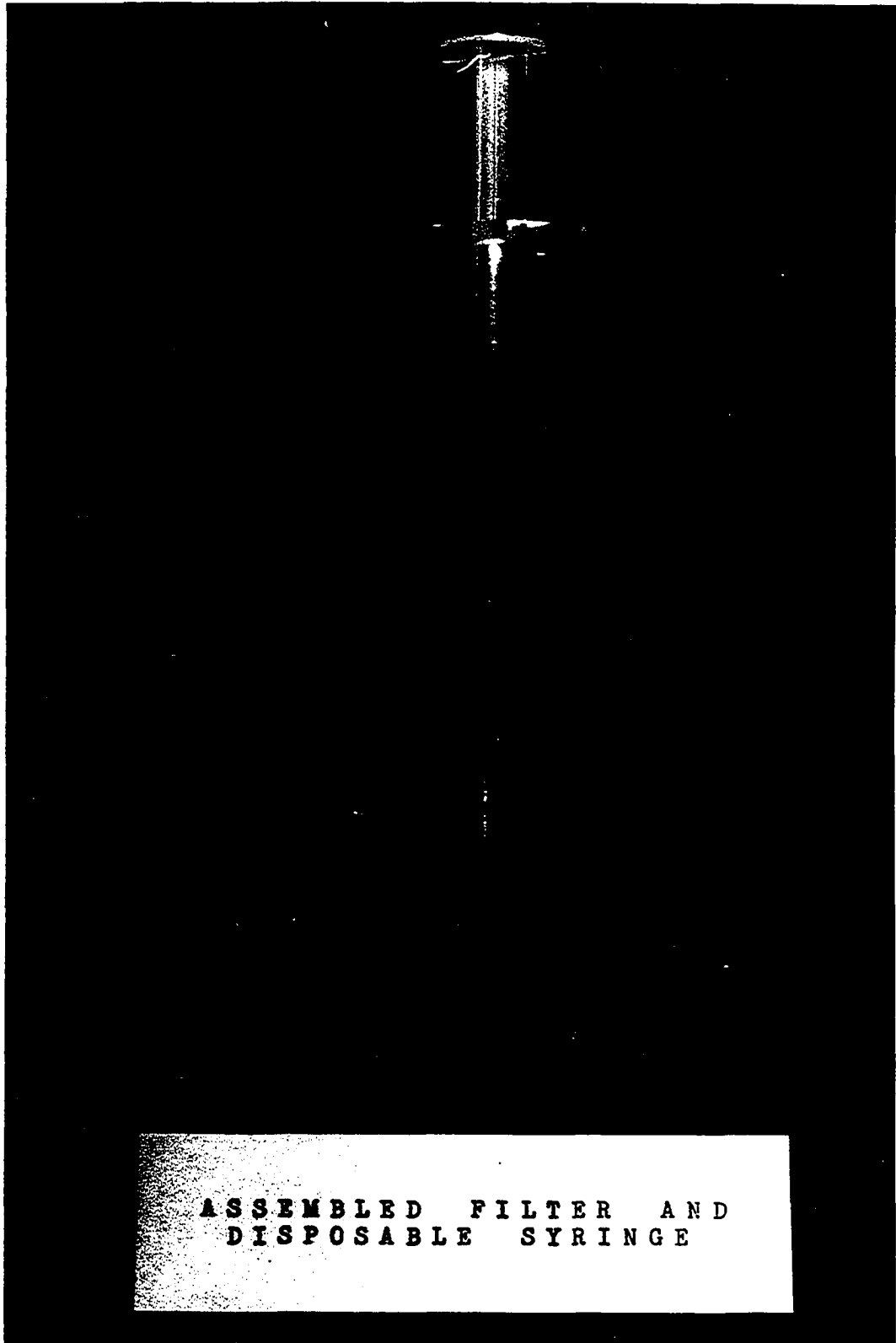
B-D VACUTAINER
NEEDLE HUB ADAPTER

figuration of syringe and filter is illustrated in Figure 6. The entire operation from filling the syringe with red blood cell suspension to filling the spectrophotometer tube required only about 5-7 seconds.

The filter system just described could be relied upon to effect complete cell removal, provided that the volume and rbc concentration of the suspension were within proper limits. Erythrocyte concentrations in excess of 8% tended to give trouble in the form of incomplete removal of cells and excessive back pressure. At 8% rbc up to 3 ml of suspension could be filtered with very few cells passing the filter. At lower concentrations 100% separation was assured. The effectiveness of cell removal was checked by direct microscopic examination of the last drop of filtrate. The most important factor to be controlled was the condition of the new filter pad. This glass fiber pre-filter is quite sensitive to mechanical abuse and filter pads must be inspected for cracks and visible imperfections before they are used.

The removal of all erythrocytes by filtration is equivalent to stopping the hemolysis. This is precisely the effect that was desired and the fact that this can be accomplished in a few seconds is quite impressive. But there is still another desirable feature of the filtration technique; the dilution effect of the addition of cold citrate - saline quench is eliminated since no quench is necessary. This advantage is of greatest influence where the absorbance is small with respect to the blank, i.e., those aliquots taken closest to time zero and therefore most significant to initial kinetics measurements. These features certainly make the filtration technique most attractive for the objectives of this study. The

Figure 6. Complete syringe and filter assembly as used to remove red blood cells from suspension.



real utility of a method, however, is also influenced by the difficulties encountered in its use. This area must now be explored.

Filter performance A volume of 3 ml of rbc suspension passed through the assembled filter will yield approximately 1 ml of a cell-free filtrate. The remainder of the liquid is absorbed by the filter pad or held in the dead space of the filter case. Such a limited amount of filtrate dictated a change in the spectrophotometric equipment, so that the smaller volumes could be dealt with directly. The Spectronic 20 used up to this time was not equipped to handle very small cuvettes. The spectrophotometric assay work was therefore transferred to a Coleman Jr. spectrophotometer¹, equipped with a Coleman 6-106, 12x75 mm round cuvette adapter modified as described in Materials and Methods.

The 7 mm cuvette tubes, chosen to allow direct spectrophotometry of the undiluted filtrate, were also a mechanical aid to the sampling technique. A wood block was drilled to support a number of these tubes sufficient to perform one complete set of timed sample aliquots. A filter, supported by the B-D VacuTainer needle hub adapter, was placed in each tube. This arrangement held the filters in position for insertion of the syringe and for delivery of the filtrate into the tube.

Beer's Law compliance This spectrophotometric set-up, in conjunction with the ability of the filters to handle rbc concentrations up to 8%, raises the possibility that a very wide range of light absorbance will be

¹Coleman Instruments Inc., 42 Madison St., Maywood, Illinois

encountered in the various hemolysis filtrates. The real variable in the temporal progress of hemolysis is the concentration of free hemoglobin. The absorbance of the filtrates is a function of the concentration but the function may or may not be linear, i.e., conform to Beer's Law. Deviation from Beer's Law is often observed in colored solutions, particularly at increasing concentration of the light-absorbing specie. The levels of free hemoglobin in these experiments may well be in the non-linear range. The following experiment was carried out to determine the log %T vs. concentration curve for hemoglobin concentrations equivalent to those used in the kinetics experiments.

Chicken erythrocytes were harvested and washed in the usual manner. The washed, packed cells were taken up to approximately 10% by volume with distilled water. The suspension was subjected to 2 freeze-thaw cycles to completely hemolyze the cells. The erythrocyte membranes ("ghosts") were sedimented by centrifugation and the clear, red supernatant was harvested for preparation of a graded series of colored solutions.

The free hemoglobin was pipetted into 10 ml volumetric flasks and diluted with distilled water to prepare a graded series of dilutions covering the entire range of hemoglobin concentrations which may be encountered in the experimental work. For greater sensitivity to possible deviation from linearity, a Beckman DU spectrophotometer¹ with regulated AC power supply was used to determine percent transmittance. This spectrophoto-

¹ Beckman Instruments Inc., 2500 Harbour Blvd., Fullerton, Calif., 92634

meter was equipped with photomultiplier; the power supply and monochromator controls were set as follows:

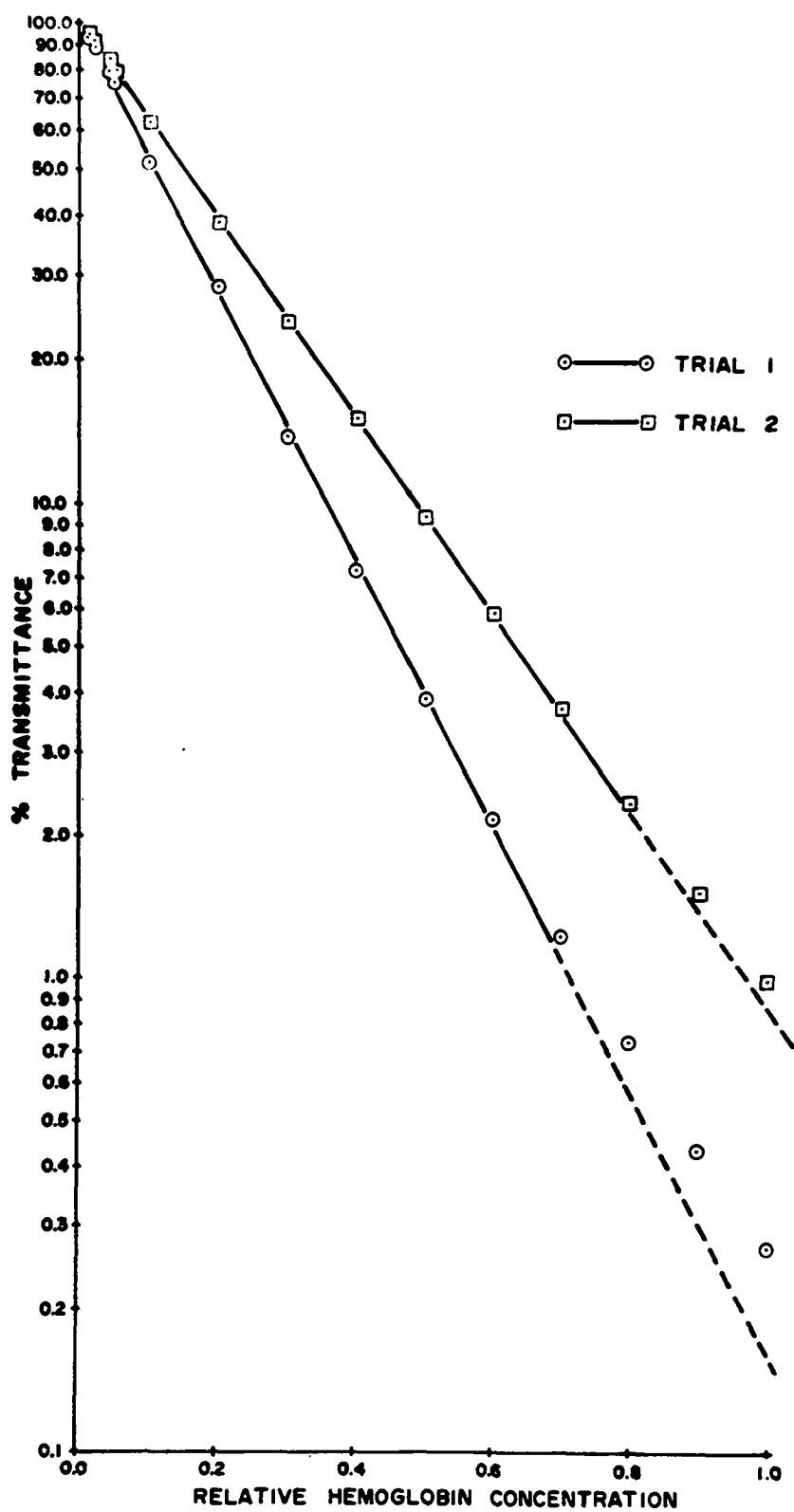
Sensitivity	1
Screen bias	5
Zero suppression	2
Slit width	0.15
Lamp	Tungsten
Wave length	540 millimicrons

Matched, square, silica 1 cm light path cuvettes were used. When T was less than 10%, the 10 x multiplier circuitry was used to expand the scale. The above experiment was carried out twice starting with freshly harvested chicken erythrocytes. The results of these two experiments are given in Table 6 and Figure 7. The graph shows that excellent Beer's Law obedience

Table 6. Relative hemoglobin concentration versus absorbance (expressed as %T)

Relative hemoglobin concentration	Trial no. 1 %T	Trial no. 2 %T
1.0	0.27	0.99
0.9	0.43	1.51
0.8	0.73	2.36
0.7	1.22	3.71
0.6	2.17	5.93
0.5	3.89	9.5
0.4	7.3	15.1
0.3	13.9	24.2
0.2	28.4	38.8
0.1	51.5	62.2
0.05	75.4	78.8
0.04	79.7	84.4
0.02	88.8	91.9
0.01	93.8	95.6
0.005	-	97.7

Figure 7. Beer's Law Test. Relative hemoglobin concentration versus log % transmittance. Spectrophotometer: Beckman DU.



is obtained to T values of 3% or less, i.e., absorbance in the range 0.00 to 1.5 gives a straight line when plotted vs. relative concentration.

Since all experimental work will be done within this range, total hemolysis controls with absorbance greater than 1 are diluted before spectrophotometric measurement is recorded. The direct proportionality of concentration to absorbance is applicable. The divergence of the two curves obtained in Figure 7 is attributed to natural variance in the erythrocytes from various chickens, as well as to the effect of qualitative initial dilution.

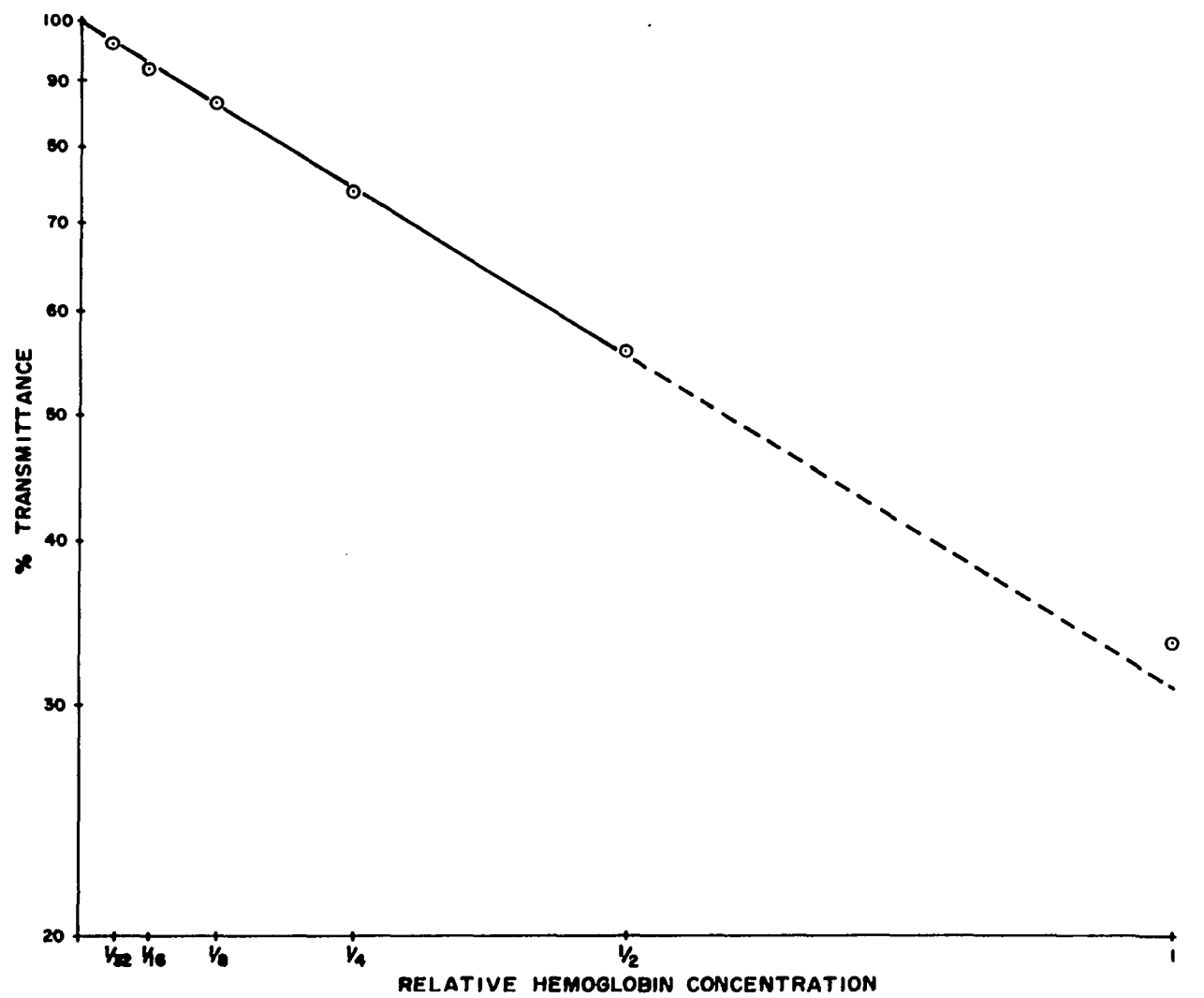
The above experiment was repeated using fewer hemoglobin dilutions to confirm Beer's Law obedience in the Coleman Jr. The 7 mm round Pyrex cuvettes prepared for the viral hemolysis studies were used for this experiment. Obedience to Beer's Law was found to be satisfactory to 50%T (Figure 8).

Filter adsorbance of hemoglobin Passing the hemoglobin solution through a bed of glass fibers such as the Millipore AP2502500 pre-filter leaves open the possibility that molecules of hemoglobin will be adsorbed onto the fibers and thus alter the absorbance of the filtrate. The following experiment was carried out to determine the extent of this effect.

The hemoglobin solutions used in the Beer's Law experiment depicted in Figure 8 were also passed through the Millipore glass fiber pre-filters. Three ml of the hemoglobin solution was drawn into a 5 cc disposable syringe¹, and expelled through the Millipore filter into a 7 mm round

¹American Hospital Supply Co., 1210 Leon Place, Evanston, Ill.

Figure 8. Beer's Law Test. Relative hemoglobin concentration versus log % transmittance. Spectrophotometer: Coleman Jr.



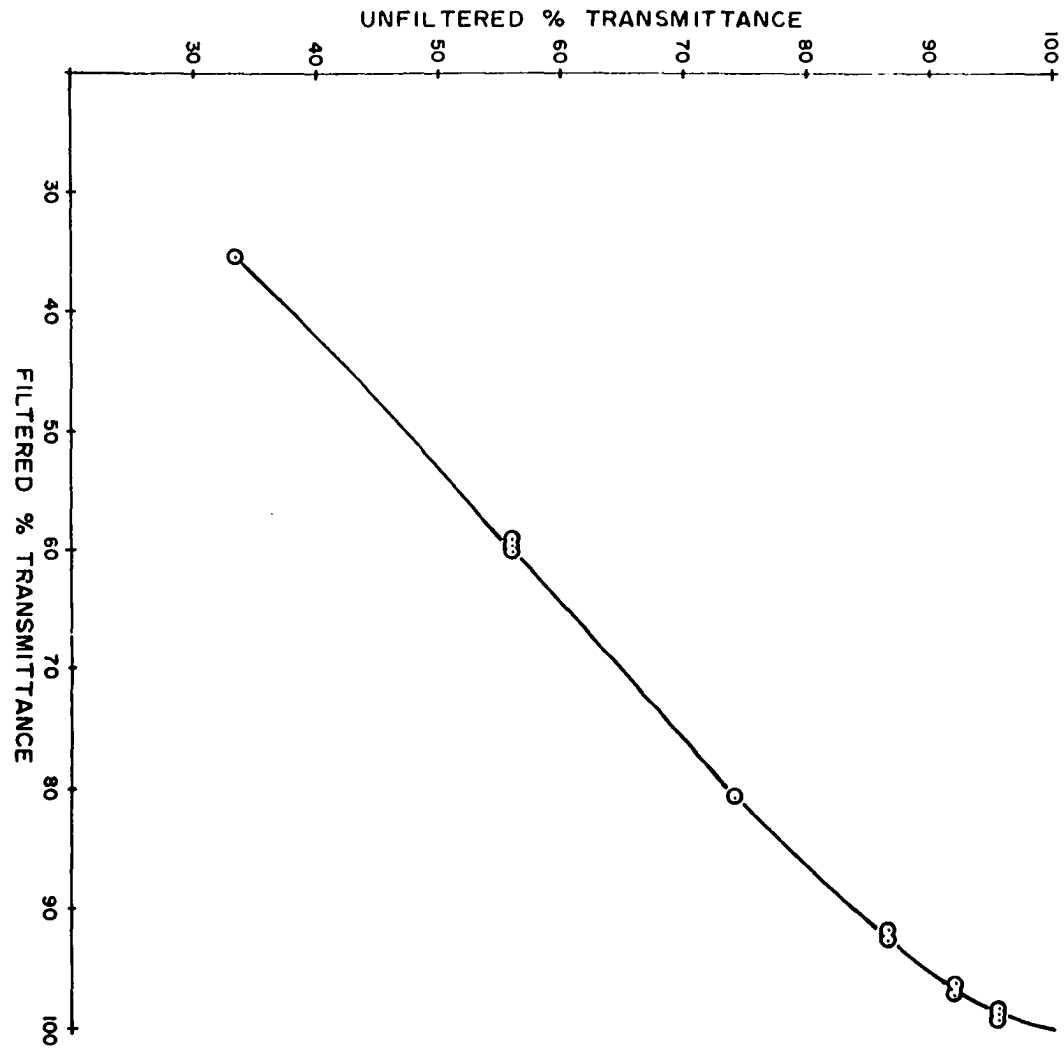
cuvette for use in the Coleman Jr. spectrophotometer. Three separate samples of each hemoglobin dilution were each filtered in this manner for triplicate determinations. The data obtained (Table 7) show that some hemoglobin is removed from the solution; therefore a correction curve was plotted (Figure 9) so that the %T of the filtrates could be adjusted to the non-filtered value whenever quantitative interpretation was desired.

Table 7. Effect of filtration on absorbance at various levels of hemoglobin concentration

Relative hemoglobin concentration	Unfiltered hemoglobin concentration	Filtered hemoglobin concentration			
1	33.5%T	35.5%T	35.5%T	35.5%T	35.5%T
1/2	56.0	59.0	60.0	59.5	
1/4	74.0	80.5	80.5	80.5	
1/8	86.5	91.5	91.5	92.5	
1/16	92.0	96.0	97.0	97.0	
1/32	96.0	99.0	98.5	98.0	

The above procedure was also useful to determine the precision of the filtration method. The variation in absorbance of triplicate filtrates of the same hemoglobin solution can be seen in Table 7 which lists the values plotted in Figure 9. The variance in transmittance of replicate filtrates of a given hemoglobin solution was found to be $\pm 0.5\%T$ from the median value. It must be recalled that the cuvettes were matched no better than this, so that the contribution of the filtration method to the absorbance uncertainty is negligible in these cuvettes.

Figure 9. Filtered % transmittance versus unfiltered % transmittance. Correction for filter adsorption of hemoglobin.



Elimination of hemolytic "drift" The percent hemolysis versus time curves plotted in Figure 4 do not appear to rise smoothly from the origin as they were expected to do. The anomalous shape of these curves was attributed to continuing hemolysis after thermal quench. The new filtration technique provides an opportunity to perform a thorough test of this hypothesis.

Five different procedures for stopping hemolysis, i.e., by removing the hemolyzing red blood cells, were tried using the same virus preparation and erythrocyte suspension. Four of the procedures involved the use of centrifugation to remove the cells with the following variations:

(1) Aliquot from beaker, quench, centrifuge

In a 150 ml beaker immersed in the 37°C water bath was placed 27 ml of 7% rbc kept agitated by a magnetic stirrer. At time zero, 3 ml of hemolytic NDV ($L.D._{50} = 10^{-9.22}$) at 37° was added and 2.0 ml samples were removed by the syringe pipet (Figure 2) at pre-determined intervals. Each sample was expelled into a chilled (0°C) test tube containing 2.0 ml of citrate-saline. After agitation in the ice bath for 30 seconds, each tube was immediately transferred to the centrifuge and spun at 800 \underline{g} for 120 seconds. As soon as the rotor stopped a sample of the supernatant was transferred to a 7 mm spectrophotometer tube.

(2) Aliquot from beaker, quench, hold, centrifuge

The procedure was identical to (1) except that the quenched tubes were left in the ice bath until all the samples (5) were taken from the run. Thirty seconds after the last sample was added to the tube

of cold citrate-saline the entire group of tubes was centrifuged for 120 seconds. The supernatants were transferred to 7 mm spectrophotometer tubes.

(3) Individual test tubes, quench, centrifuge

The 7% rbc suspension was dispensed into separate 13x100 mm test tubes, each containing 1.8 ml of the suspension. A rack of 5 of these tubes was incubated at 37°C and to each tube was added 0.2 ml hemolytic NDV. Hemolysis was allowed to proceed until the programmed time had elapsed. The tube was then removed from the 37°C bath, plunged into a 0°C bath and 2.0 ml of 0°C citrate-saline was added to the tube to hasten the quench. The tube was held in the ice bath for 30 seconds, centrifuged for 120 seconds and the supernatant transferred at once to a 7 mm spectrophotometer tube.

(4) Individual test tubes, quench, hold, centrifuge

The protocol was similar to (3); virus and red blood cells were mixed in individual tubes. Quenched tubes were held in the ice bath until the end of the run, at which time all were centrifuged.

(5) Aliquot from beaker, filter, dilute

The fifth procedure used filtration to stop the reaction. A beaker containing 27 ml of 7% rbc was immersed in the 37°C water bath. During the course of the run the cells were kept suspended by a magnetic stirrer. At time zero, 3.0 ml of 37°C hemolytic virus suspension was added to the beaker. A few seconds before the selected time elapsed, approximately 3 ml of the mixture was aspirated into a 5 cc disposable hypodermic syringe. The syringe was attached to a

filter and at exactly the selected time the filtrate was expelled into a 7 mm spectrophotometer tube.

All of the above procedures were carried out in triplicate. Blanks and controls (100% hemolysis) were prepared by methods appropriate to each procedure. The resulting supernatants and filtrates were read in the Coleman Jr. spectrophotometer using distilled water as 100%T for the blank and the blank as 100%T for all hemolyzed specimens. Since the filtrates were not diluted as was the case with the quenching procedure, spectrophotometry was carried out on both undiluted and 1:2 diluted (with citrate-saline) filtrates. The results of this experiment are tabulated in Table 8. Figures 10 and 11 convert these data to graphical form for visualization of their significance.

The data of Table 8 are presented graphically as log %T versus time in lieu of percent hemolysis versus time. Percent hemolysis was defined as

$$\% \text{ hemolysis} = \frac{\text{absorbance at time } t \times 100}{\text{absorbance of total hemolysis}} .$$

But the absorbance (absorbance = $-\log \%T$) of a given totally hemolyzed control is a constant, hence % hemolysis is proportional to absorbance. Under this definition the curves of percent hemolysis versus time and absorbance versus time are identical except for a proportionality constant affecting the ordinate axis. The same relationship holds for percent hemolysis versus time and log %T versus time except that the proportionality constant is negative, i.e., the direction of curvature is reversed.

Table 8. Centrifugal vs. filtration, evaluation for application to initial kinetics of NDV-induced hemolysis (%T of blanks vs. distilled H₂O, %T of all other samples vs. corresponding blank)

Hemolysis time(sec.)	Aliquot from beaker quench-centrifuge				Aliquot from beaker quench-hold-centrifuge			Aliquot from beaker filter-undiluted		
	%T	%T	%T	%T	%T	%T	%T	%T	%T	%T
0"(blank)	95.0	94.5	94.0	95.0	94.0	94.0	94.0	87.5	89.0	90.0
∞"(saponin)	5.0	5.0	4.5	5.0	5.0	5.0	5.0	3.5	3.0	3.0
40"	95.0	96.0	96.5	-	89.5	90.0	84.5	100.0	100.0	100.0
80"	95.0	93.5	95.5	93.0	83.0	82.0	75.0	98.0	96.0	96.5
140"	87.0	80.5	86.0	88.0	75.0	67.0	69.0	88.0	88.0	87.0
190"	78.5	73.0	79.0	76.5	72.5	73.0	65.0	73.0	75.0	71.0
240"	72.0	62.0	66.0	66.0	67.0	70.0	61.5	-	54.0	56.0

	Individual test tubes quench-centrifuge			Individual test tubes quench-hold-centrifuge			Above filtrate 1:2 dilution		
	%T	%T	%T	%T	%T	%T	%T	%T	%T
0"(blank)	87.0	86.5	88.0	92.0	91.0	92.0	93.5	94.5	94.5
∞"(saponin)	5.0	5.0	5.0	4.0	5.0	5.0	5.0	5.0	5.0
40"	94.5	95.0	95.0	78.0	77.5	76.0	100.0	99.5	100.0
80"	90.0	89.0	87.5	67.0	69.5	69.0	98.5	97.0	99.0
140"	85.5	83.0	82.0	61.0	61.0	60.0	95.0	93.5	93.5
190"	75.0	76.5	76.5	63.5	62.0	61.5	85.5	87.0	85.0
240"	73.0	73.5	78.0	65.0	66.5	63.0	-	73.5	76.0

Figure 10. Comparison of two techniques for "stopping" hemolysis.

- A. Aliquot from beaker, quench technique (procedure (1), pages 88, 89, and procedure (2), pages 88-89).
- B. Aliquot from beaker, filtration technique (procedure (5), pages 89-90).

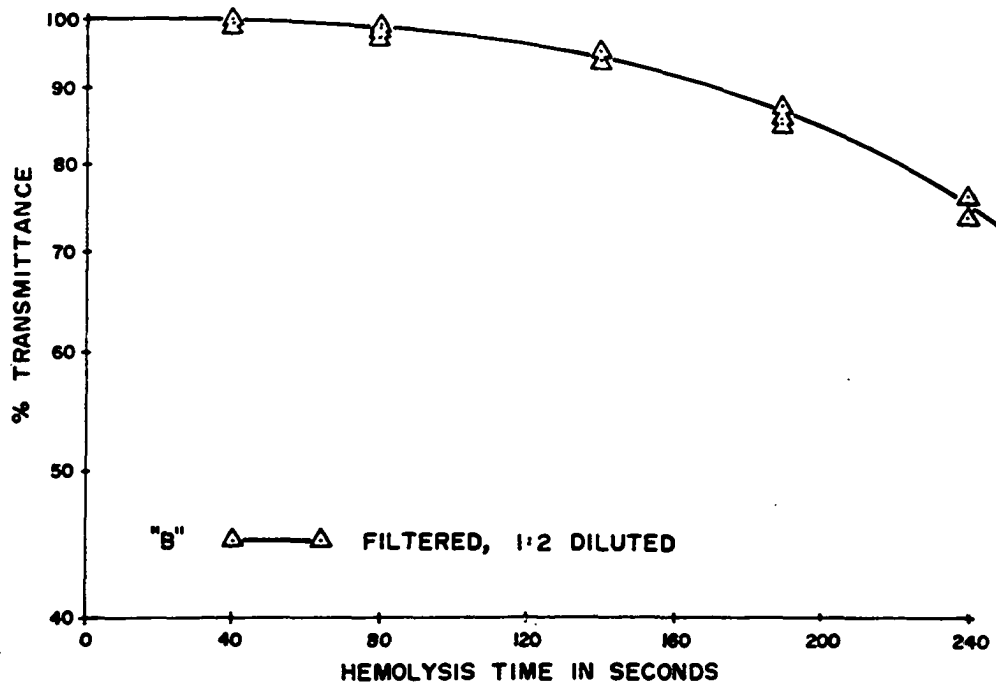
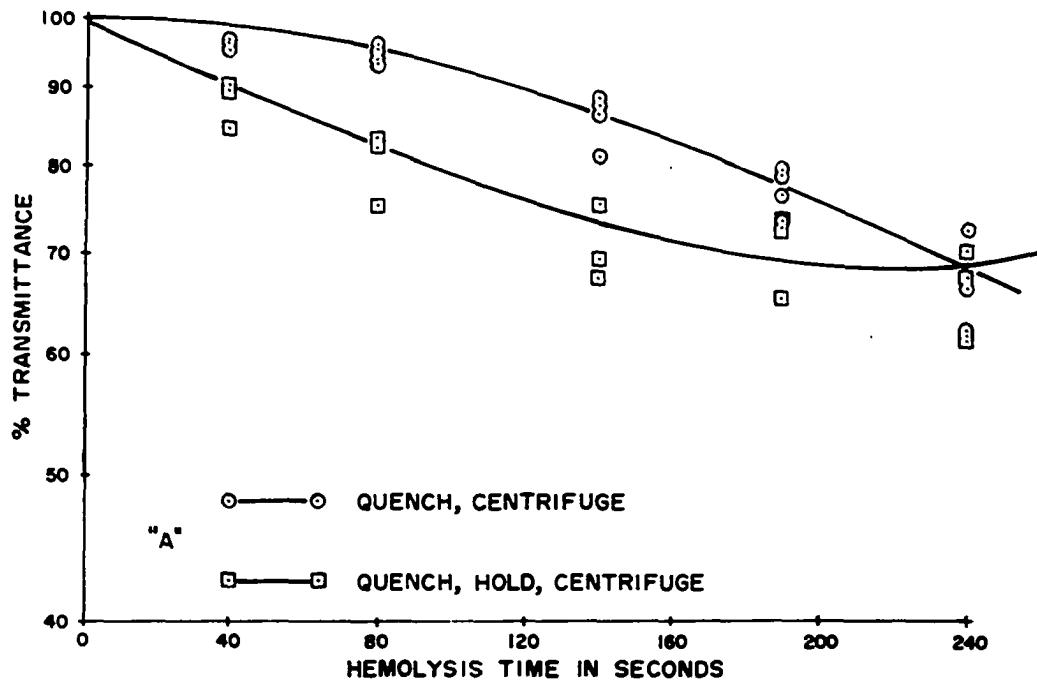
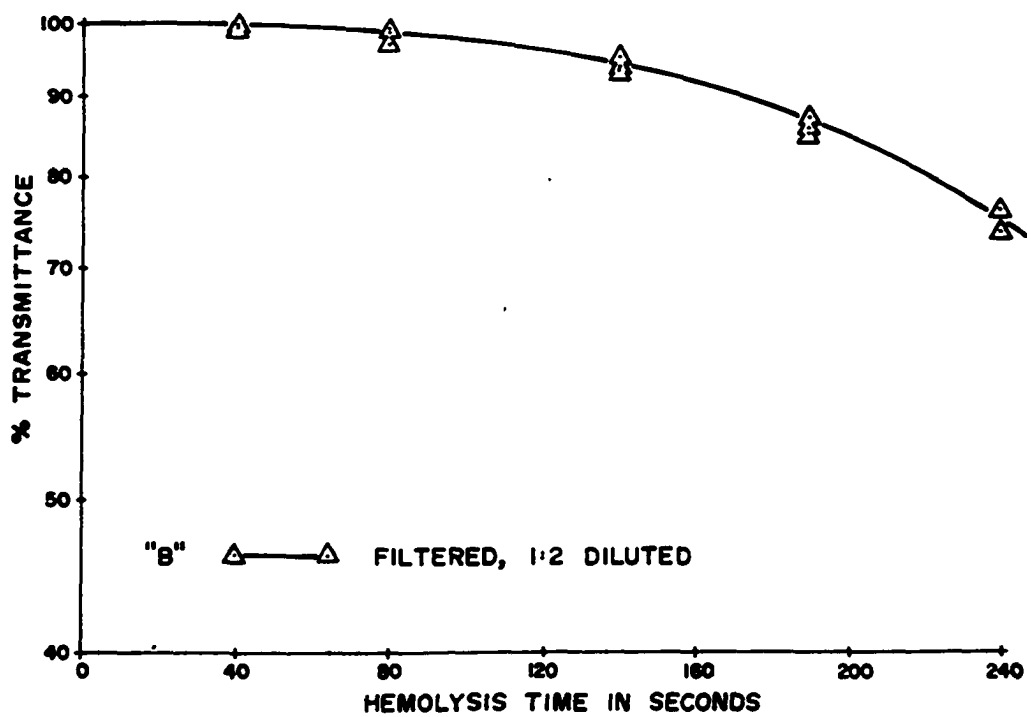
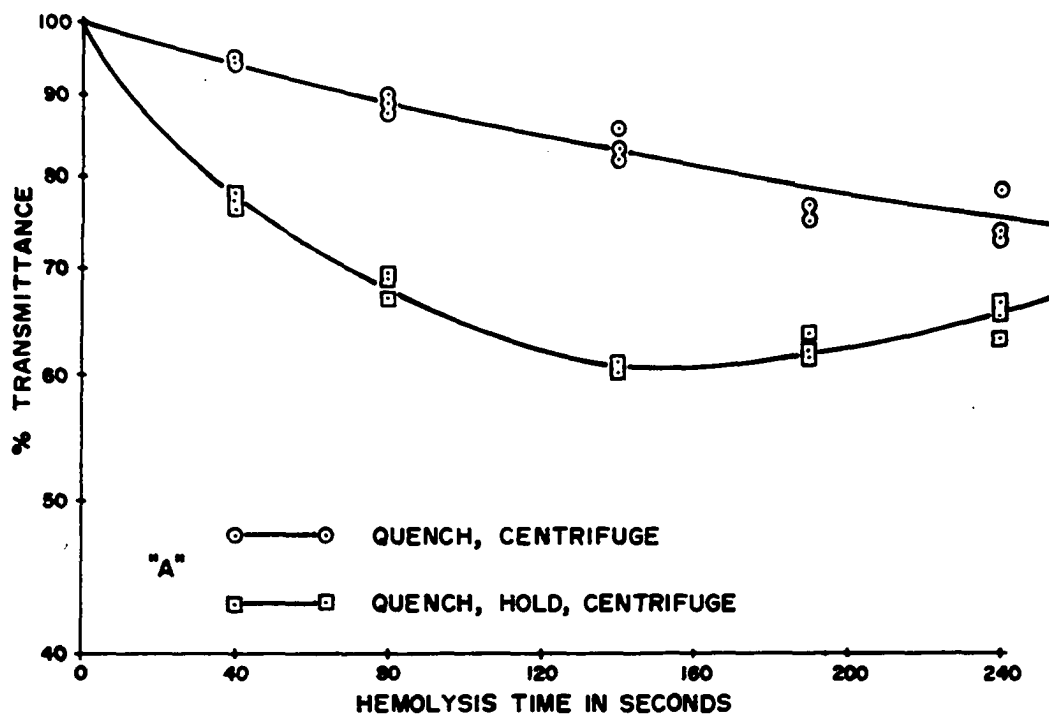


Figure 11. Comparison of two techniques for "stopping" hemolysis.

- A. Individual test tube, quench technique (procedures (3) and (4), page 89).**
- B. Aliquot from beaker, filtration technique (procedure (5), pages 89-90).**

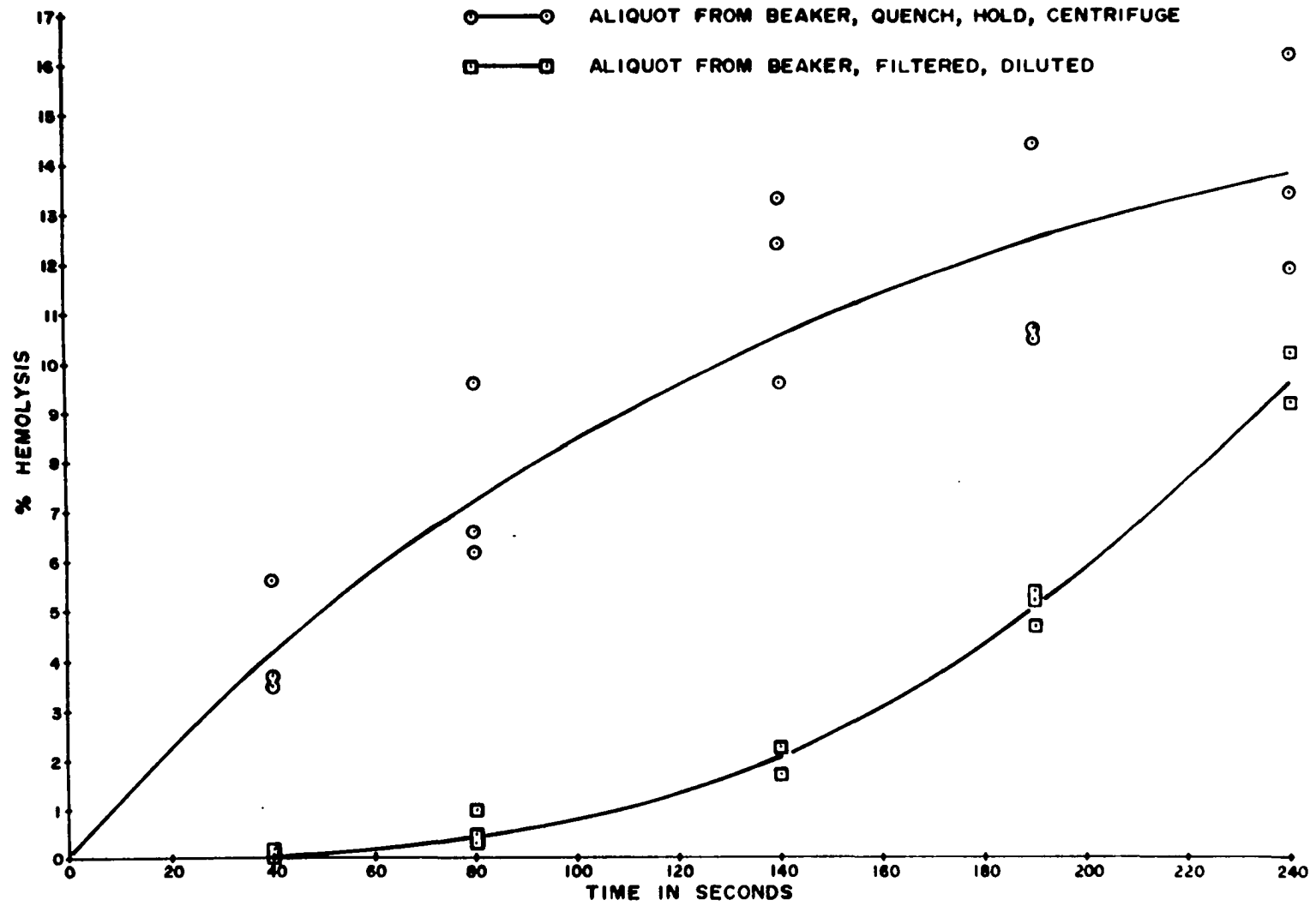


This property is illustrated in Figure 12 with two sets of data (from procedures (2) and (5)) from the previous experiment. Percent hemolysis is plotted versus time in Figure 12 where it may be noted that each curve is a reverse image of the corresponding data plotted in Figure 10. Since the forms of presentation are equivalent, future graphs of kinetic data will be presented in the form $\log \%T$ versus time or absorbance versus time. This form has a great advantage at high concentrations of rbc where it is difficult to achieve total hemolysis.

The curves of Figures 10 and 11 are dramatic evidence of the continuing hemolysis during the quench-centrifuge-decant procedure. This is especially the case when all the samples of a single run are held in the ice bath until they can be centrifuged as a group. The effect of hemolytic "drift" is so marked in this case that the true shape of the $\log \%T$ - time curve is lost entirely. The transmittance of the supernatant must either decrease or remain constant with time during hemolysis due to the irreversibility of this process. Furthermore, the strenuous efforts expended in carrying out the centrifugation as quickly as possible in this one experiment are far from practical as a routine. This comparison of filtration and centrifugation techniques may therefore be somewhat conservative in the differences it shows.

Figure 12 is also useful for direct comparison with the data presented in Figure 4. Although there were some differences in experimental details, the hypothesis of hemolytic "drift" continuing after quenching seems to be correct. The anomalous portions near the origins of the curves in Figure 4 are probably due to the uncontrolled timing of centrifugation

Figure 12. Data from procedure (2), pages 88-89 and from procedure (5), pages 89-90, recalculated to show percent hemolysis versus time.



and decantation of the supernatant in that experiment. At that time hemolytic "drift" was not recognized as an interfering factor. This problem has been eliminated, however, by the filtration technique without any apparent sacrifice and perhaps even some improvement in repeatability. This latter observation may be made from the five sets of data of Figures 10, 11 and 12. Further experiments will show whether these features of the filtration technique are beneficial or misleading.

Reproducibility and repeatability of experimental procedure The

reproducibility and repeatability of the filtration technique may be further tested by using it to perform two identical experiments, each commencing with the collection of fresh erythrocytes and the preparation of hemolytic 174HS from the frozen pool. A 150 ml beaker containing 44 ml of 3.0% rbc was immersed in the 37°C water bath. The cells were kept in suspension by just-adequate mechanical stirring. The hemolytic virus, 9 ml in a 25 ml graduate, was incubated in the same bath. While temperature was stabilizing, 4.0 ml of the cell suspension was drawn from the beaker with a 5 cc syringe. Citrate-saline was added to the contents of the syringe until the volume was 5 ml. This sample was reserved for the blank. A similar sample was drawn using distilled water as diluent, to which a small amount of saponin was added for total hemolysis control. After temperature equilibration the prewarmed virus was added to the remaining rbc suspension (36 ml) and the timer was started. Samples were drawn by a syringe at selected time intervals and expelled through a filter into a 7 mm spectrophotometer tube. This procedure was carried out in triplicate

to establish repeatability. A fresh supply of erythrocytes was harvested, fresh hemolytic virus was prepared from the frozen pool and the experiment was repeated from the beginning to establish reproducibility. The results are given in Table 9. The data were averaged for each time interval for each experiment in Table 9, so that the curves could be plotted together without a possibly confusing number of data points. These average values are shown in Figure 13. The two curves agree very well; reproducibility appears to be satisfactory despite the fact that the repeatability (Table 9) is rather disappointing. At this time it is difficult to account for the scatter in the replicate data. The previous experiments indicate that the filtration technique is probably not responsible for the observed variation.

The sigmoid tendency of the curve in Figure 13 is not a stranger to hemolytic phenomena. Ponder [60], in his chapter "Kinetics of Hemolysis" (pages 169-171), describes percentage hemolysis vs. time curves as "typically sigmoid". Recognizing that Ponder's discussion concerns human erythrocytes and that the experimental data of Figure 13 represent only a portion of the total hemolysis curve, another experiment was executed with a 5% concentration of rbc and covering a longer time interval so that the shape of the curve could be revealed more completely. The results of this experiment are shown in Figure 14 in which the character of this log %T vs. time curve is clearly sigmoid. This observation is intuitively satisfying and may be interpreted as reflecting a heterogeneity of red blood cell resistances to the lysin. This position has been defended by Ponder and Cox [61] for chemical lysins such as saponin. It is plausible

Table 9. Character and reproducibility of log(%T) versus time curve for NDV-induced hemolysis

Time (seconds)	Trial no. 1			Trial no. 2		
	TriPLICATE - %T			TriPLICATE - %T		
	Infectivity titer = $10^{-9.5}$			Infectivity titer = $10^{-8.8}$		
0" (blank) ^a	96.8	97.0	96.5	95.0	95.1	94.1
5"	100.0	99.4	100.0	100.0	100.0	100.0
20	100.0	99.0	100.0	100.0	99.0	100.0
40	100.0	97.7	99.3	100.0	99.0	98.1
60	98.0	92.8	95.2	99.7	97.0	92.5
80	93.9	94.2	92.0	97.1	93.3	86.0
100	86.4	91.1	89.1	93.8	89.2	88.0
140	-	76.0	-	84.8	81.9	82.1
180	67.9	62.2	63.8	77.0	73.0	73.9
240	60.9	53.4	54.1	60.1	51.7	56.0
300	45.0	40.0	41.0	43.5	41.2	37.1
450	26.5	28.0	25.3	29.3	27.1	27.9
600	19.8	18.8	17.1	22.0	20.1	21.5
∞ (control)	4.0	4.0	4.1	3.5	3.7	3.4

^aBlank %T versus distilled water, all other values versus corresponding blank.

Figure 13. Character and reproducibility of log % transmittance versus time curve.

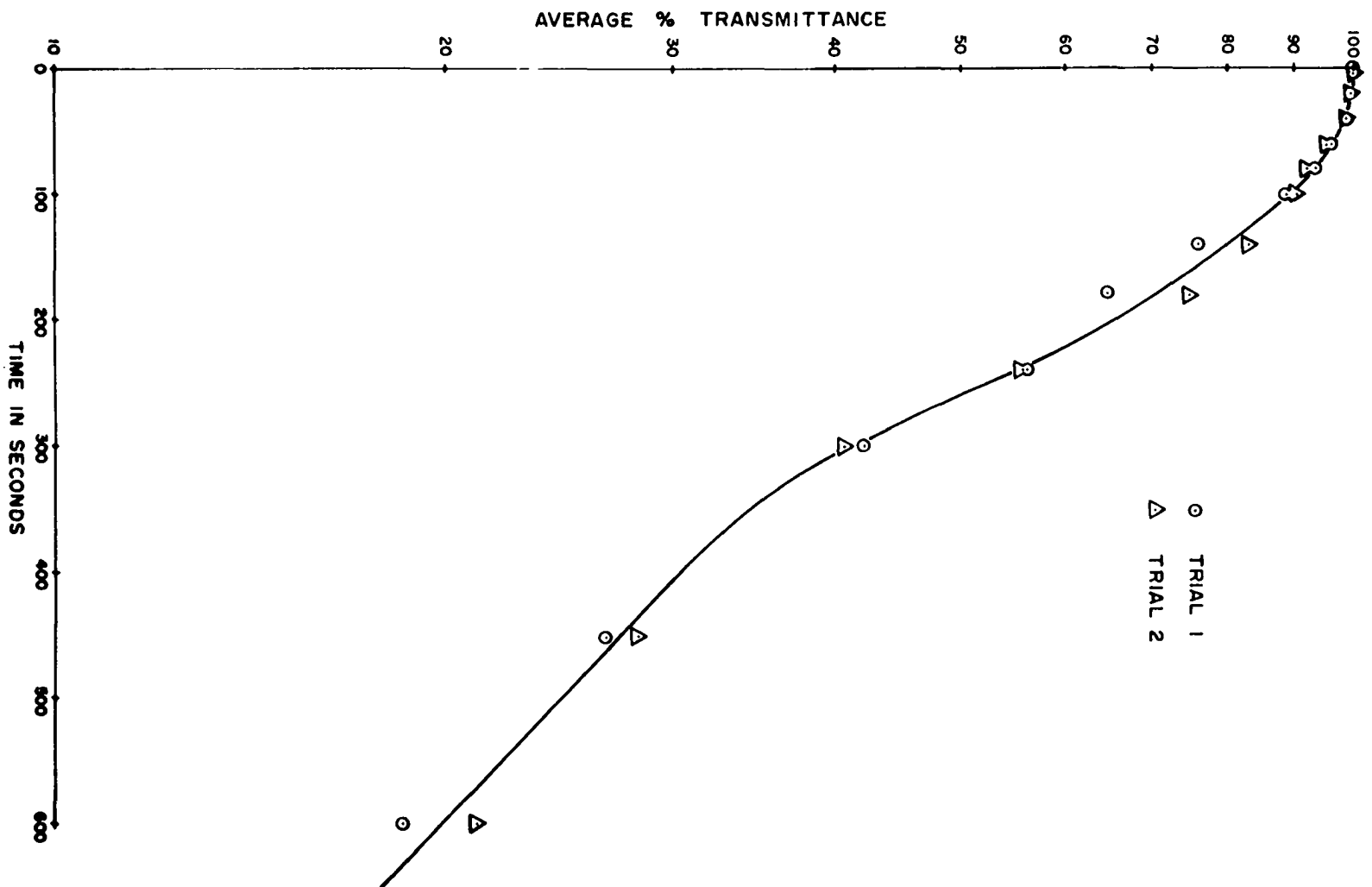
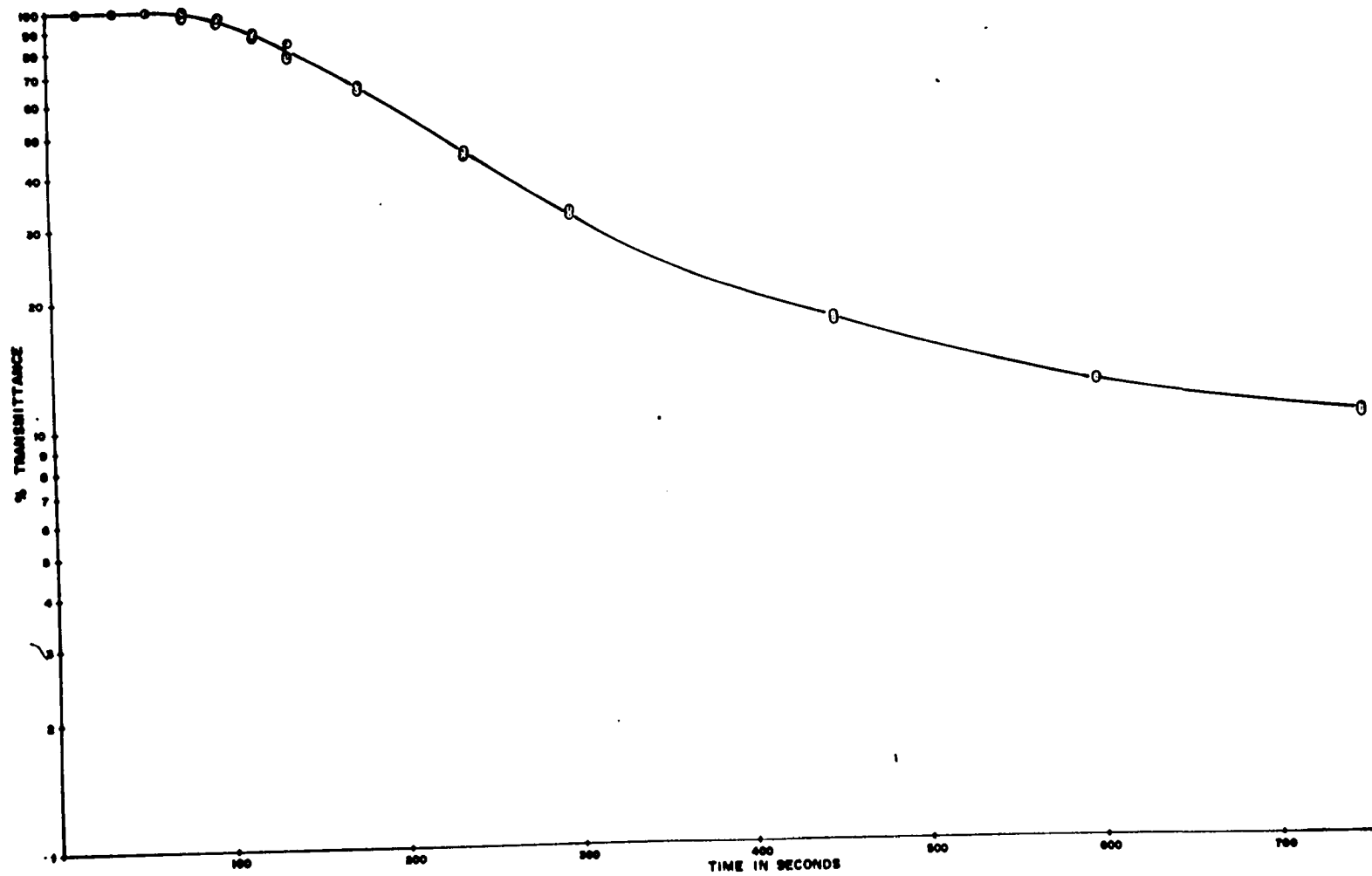


Figure 14. Sigmoid character of NDV-induced hemolysis (log % transmittance) versus time curve. Substrate: 5.0% chicken erythrocytes; virus infectivity titer: L.D.₅₀ = $10^{-8.75}$.



that the chicken erythrocyte population is also heterogeneous in resistance to the hemolytic attack of NDV. Such heterogeneity is expected to explain the sigmoid hemolysis rate curve that was observed using the filtration technique (Figure 14).

Validity of experimental procedure A final check on the suitability of the filtration technique was performed by treating separately both rabbit erythrocytes and chicken red blood cells with the same hemolytic preparation of NDV. Rabbit rbc are resistant to hemolysis by NDV and mumps virus [14, 18]. If the filtration technique is yielding unbiased data, the rabbit cells should exhibit the expected immunity to hemolytic attack since the hemolysin is NDV as was used by Burnet and Lind [14]. Rabbit erythrocytes, obtained by cardiac puncture, were washed and diluted in a manner identical to that used to prepare the chicken rbc suspensions. The suspensions were diluted to provide a final erythrocyte concentration of 5.0%. Hemolytic NDV was prepared as usual. Forty-five milliliters of each cell suspension was incubated to 37°C before addition of 5 ml of the virus inoculum at 4°C. Blanks and total hemolysis controls were prepared in the usual way. Samples were taken via syringe and filtered for spectrophotometry. The results presented in Table 10 and Figure 15 show that the expected immunity of rabbit erythrocytes to NDV hemolysis was observed.

The filtration technique has now been shown to be rapid, reproducible, quantitative and, by at least one criterion, unbiased. The ease and convenience of the method contribute further to its utility. It is possible

Table 10. Comparative hemolysis rates, chicken versus rabbit erythrocytes

Time: seconds	Chicken erythrocytes %T		Time: minutes	Rabbit erythrocytes %T
0"(blank)	94.0 ^a	94.0 ^a	0'(blank)	97.0 ^a
180	91.5	89.0	3	99.0
210	-	85.0	4	99.0
240	86.0	78.0	6	99.0
260	76.0	75.0	10	99.0
280	71.5	70.0	15	99.0
300	66.5	62.5	20	98.0
330	61.0	56.0	30	99.0
360	59.5	56.0	40	99.0
400	50.0	51.0	50	98.0
450	46.0	43.0	60	97.0
500	41.0	40.0	75	96.0
560	37.5	36.5	∞(saponin)	9.0
650	31.5	32.0		
750	-	27.0		
795	21.5	-		
∞(saponin)	3.5	3.5		

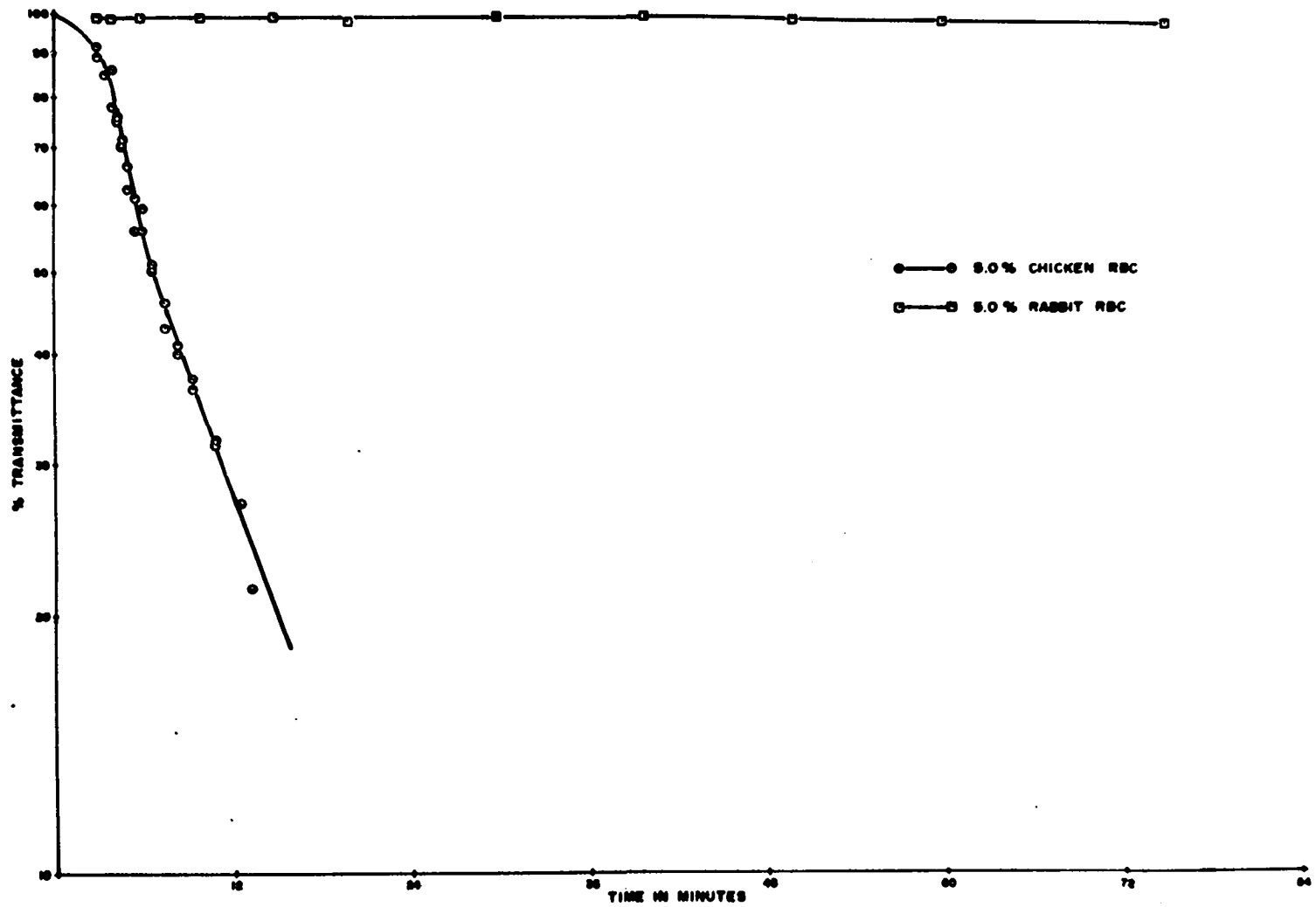
^aTransmittance of blanks measured versus distilled water. All other assays measured versus appropriate blank.

now to apply this method with confidence to the study of the initial kinetics of NDV-induced hemolysis.

Optimum erythrocyte-virus concentration

To review briefly, it is the purpose of the overall program to establish a relationship between the concentration of red blood cells and the rate of hemolysis when the concentration of hemolytic virus is fixed. Furthermore, the initial kinetics are of primary interest and these are to be obtained by the use of spectrophotometry. The technique must

Figure 15. Comparative hemolysis rates. Chicken versus rabbit erythrocytes virus infectivity titer: $L.D._{50} = 10^{-7.62}$.



measure the increase in absorbance of the hemolyzate filtrate as a function of time. At the earliest intervals the increase in absorbance of the hemolyzate filtrate is small and experimental error becomes proportionally more significant. To reduce the effect of experimental error the concentration of virus may be increased, which proportionately increases the amount of hemoglobin liberated per unit time; in effect, it increases the rate of hemolysis. On the other hand, as the rate of hemolysis increases so does the difficulty of measuring the rate accurately. Whether or not the concentration of virus can be increased without increasing the rate beyond the capability of the technique has yet to be determined.

Virus concentration There are two possible methods to increase the amount of hemolytic virus present in a hemolysis rate study. The concentration of hemolytic virus, as expressed by infectivity titer, may be held constant while the proportion added to the red blood cell suspension is increased, or the proportion may be held constant while the concentration is increased. It might be possible to concentrate the hemolytic fraction of the virus which associates with the allantoic fluid precipitate by diluting the precipitate to less than the original volume. An attempt was made to double the concentration by diluting to only one-half of the original volume. The precipitate dissolved as expected but the solution was unstable; in a short time irreversible precipitation produced fine white particles and thus rendered the pool unusable for hemolytic studies. Lucie [49] had observed the same phenomenon over a longer period of storage but with hemolytic virus in lower concentration. Apparently, in-

creasing the concentration of the virus accelerates the precipitation. Hemolytic stock solutions (strictly speaking they are suspensions) diluted to original volume were sufficiently stable that no precipitate was visible for at least 18 hours from the time they were prepared. Nevertheless, the stock solution was not prepared until immediately before use. It was observed further in this study that incubating the virus suspension at 37°C accelerates the irreversible precipitation. For this reason the hemolytic stock solution was kept refrigerated and portions were removed for incubation to 37°C as needed.

Because of the difficulty of concentrating the hemolytic NDV stock solutions any increase in hemolytic virus concentration during hemolysis must be achieved by adding a larger volume of the stock solution. It remains to be seen whether an increase will be necessary.

Erythrocyte concentration The earlier attempt to determine the level of substrate saturation for the hemolytic system was cut short by the failure of the quench-centrifugation technique. A new experiment utilizing the filtration technique will renew the effort to establish the enzyme saturation level. The relationship between reaction velocity and substrate concentration, all other parameters being held constant, is of great significance to the interpretation of a mechanism from kinetics data. Until now both virus and substrate (erythrocyte) concentrations had been chosen arbitrarily for convenience and accuracy of handling and assay. This experiment was undertaken to determine whether the 5% erythrocyte concentration used previously is sufficient to obtain a near-maximum rate of hemolysis. It was not necessary to increase the concentration of virus

for this study.

Washed chicken rbc were taken up in citrate-saline in the usual manner. Dilutions were prepared to yield 5.0%, 6.0%, 8.0%, and 9.0% rbc after addition of virus. Each trial was carried out in triplicate using 5.0 ml of 37°C hemolytic virus suspension to 45 ml of 37°C rbc dilution. The blanks and the total hemolysis controls were prepared from each dilution as usual. Results of the experiment are shown in Table 11 and Figure 16.

Figure 16 is a tracing of the original graph which used different colors to distinguish the data points for each concentration of erythrocytes. Without color differentiation it is very confusing to plot these points, especially for times less than 120 seconds where the curves lie close together. For this reason only the curves were traced and no data is shown. The Table 11 is almost sufficient in itself, however, and inspection of the data for 8.0% and 9.0% rbc shows that there is essentially no difference in the rate of increase of absorbance for filtrates from either 8.0% or 9.0% rbc. This suggests that at this concentration (1.9×10^8 infective units/ml) is sufficient to elicit the maximal hemolysis rate.

Evaluation of observed scatter Inspection of the data presented in Table 11 shows that the replicate values suffer considerable scatter (as had been noted previously). For the purpose of kinetics measurements such uncertainty in the data points creates a nearly impossible ambiguity. Measures were taken, therefore, to ascertain the causes of this variation in identical samples. Two separate factors were discovered which contributed to the observed scatter.

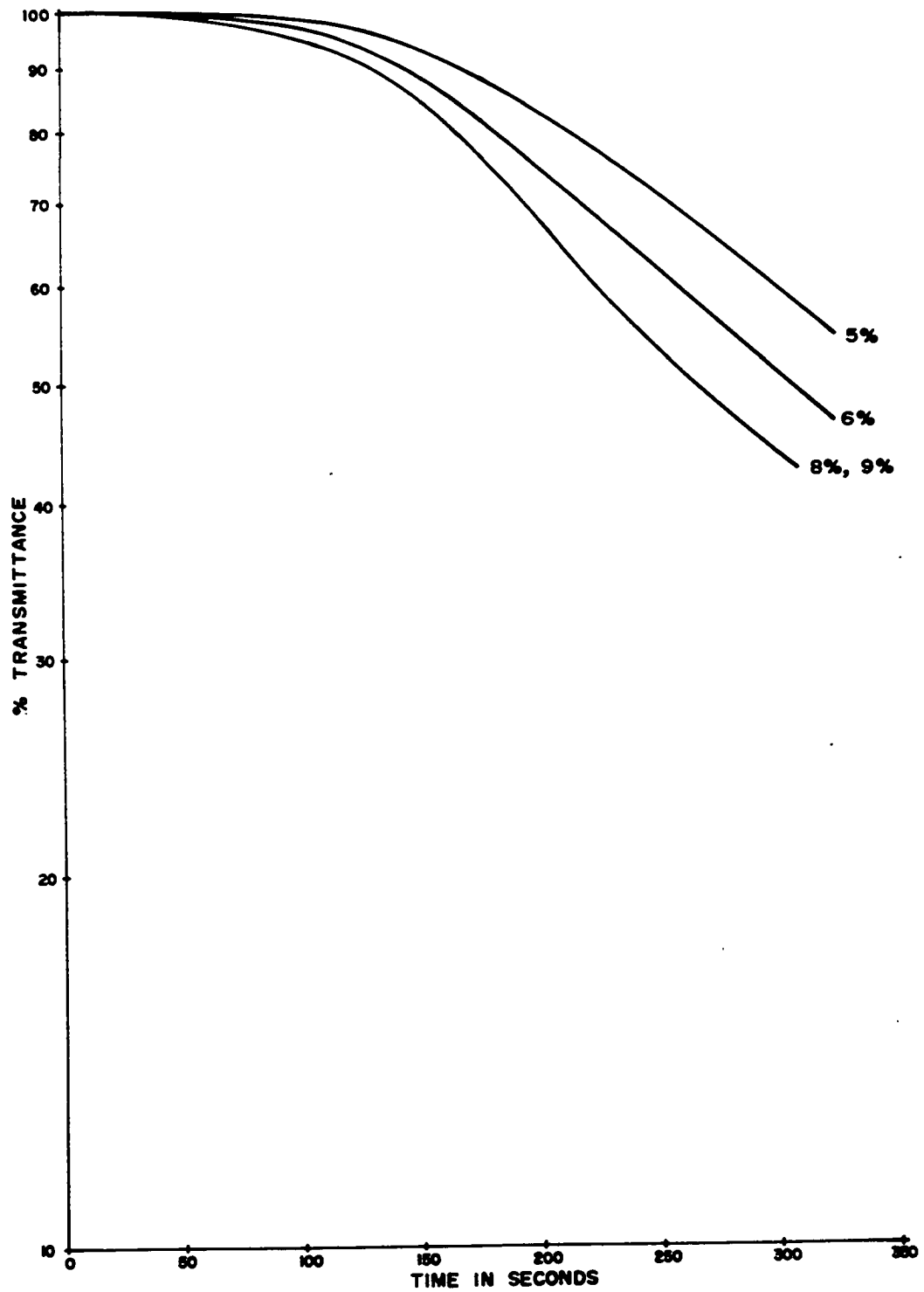
Table 11. Effect of increasing substrate (erythrocyte) concentration on %T at various times

Time seconds	5.0% rbc			6.0% rbc			8.0% rbc			9.0% rbc		
	%T	%T	%T	%T	%T	%T	%T	%T	%T	%T	%T	%T
0 (blank)	95.0	95.0	95.0	91.0	89.5	90.0	88.0	89.0	89.0	88.0	89.5	88.5
40	99.5	100.0	98.0	99.5	98.5	98.5	99.0	99.0	99.0	99.5	99.0	98.0
80	99.0	99.0	97.0	98.0	98.0	98.0	96.0	97.0	96.0	95.0	96.0	94.0
100	-	-	-	96.5	96.0	97.0	89.0	90.0	93.0	91.0	93.5	94.0
120	96.5	95.5	94.0	93.0	93.0	92.0	88.0	92.0	90.0	84.5	85.5	90.0
140	95.0	95.5	90.0	91.0	89.0	90.0	84.0	84.0	88.5	86.5	84.0	83.5
160	90.5	89.0	85.0	85.5	85.0	84.0	73.5	79.5	79.0	81.0	71.0	75.0
180	85.5	82.0	83.0	80.0	78.5	80.0	63.5	72.0	70.5	76.0	65.0	74.5
200	83.0	79.0	80.5	74.5	72.0	73.0	60.5	64.0	63.0	63.0	65.0	63.5
220	77.0	72.0	74.0	68.0	68.0	67.0	57.5	61.5	58.5	52.5	56.0	62.0
240	73.0	70.5	71.5	64.0	58.5	62.0	48.0	52.0	52.0	50.5	51.5	53.0
270	-	-	-	58.0	-	56.5	46.0	49.0	47.5	44.0	46.0	47.5
280	63.5	59.5	61.5	-	53.0	-	-	-	-	-	-	-
300	-	-	-	51.0	49.0	48.0	41.0	42.5	44.0	42.0	44.0	42.5
320	55.0	54.0	53.5	-	-	-	-	-	-	-	-	-
∞ (control)	5.0	5.0	5.0	4.0	4.0	4.0	3.5	3.5	3.5	3.0	3.0	3.0

Infectivity titer of virus: $LD_{50}=10^{-9.28}$
 All other %T vs. blank

Blank %T vs. distilled water

Figure 16. Effect of increasing substrate (erythrocyte) concentration on log % transmittance at various times.
(See Table 11 for detailed data.)



The technique of sampling the erythrocyte-virus reaction mixture was found to be a potential cause of variation in otherwise identical samples. The sampling technique involves removing sufficient erythrocyte-virus suspension so that at least 1 ml of filtrate can be obtained. The minimal sample required is in the neighborhood of 3 ml provided that 1 - 2 ml of air can be forced through the filter after the sample to force the filtrate to enter the spectrophotometer tube. When sampling with a 5 cc syringe it is convenient to take 3 ml of sample, finish filling the syringe with air and express sample and air through the filter until sufficient filtrate is transferred to the spectrophotometer tube. This air pad, however, can be a source of trouble in mechanical hemolysis during sampling. An experiment was designed to reveal a sampling technique minimizing spurious hemolysis.

Five sampling techniques were devised for comparison with one another. The techniques are as follows, 5 cc disposable syringes used throughout:

(1) No needle, draw sample, air

Luer tip of closed syringe immersed in sample, 3 ml drawn into syringe, 2 ml of air drawn into syringe allowing it to bubble through sample, sample expelled through filter.

(2) No needle, draw air, sample

Luer tip of syringe containing 2 ml of air immersed in sample, 3 ml drawn into syringe, sample expelled through filter.

(3) No needle, draw sample, air through needle

Technique similar to (1) except that 26 gauge needle placed on syringe while air was being inspired.

(4) Needle, draw sample, air

Both sample and air drawn into syringe through 26 gauge needle, needle removed and sample expelled through filter.

(5) No needle, draw sample, no air

Luer tip of closed syringe immersed in sample, 4 ml of sample drawn into syringe, no air, sample expelled through filter.

Two comparisons of the five techniques were carried out. In one case, 4 samples were drawn from the same pool of erythrocyte suspension. Results from this experiment indicate the repeatability of each technique. In the second case, from a single pool, one sample was taken by each of the five techniques. This procedure was repeated 4 times to establish reproducibility. The results indicate which of the five techniques is least traumatic to the red blood cells. Both of these experiments utilized approximately 5% rbc suspensions prepared as usual for hemolysis study. The data are presented in Tables 12 and 13.

Table 12. Repeatability experiment comparing five sampling techniques

Technique	Quadruplicate samples for repeatability			
	%T	%T	%T	%T
(1) No needle, draw sample, air	91.5	90.5	87.5	84.5
(2) No needle, draw air, sample	83.0	-	81.0	80.5
(3) No needle, draw sample, air through needle	93.5	91.0	95.0	93.0
(4) Needle, draw sample, air	85.5	83.5	73.0	89.0
(5) No needle, draw sample, no air	96.0	96.0	92.0	95.5

All values versus distilled water as 100%T

Table 13. Hemolytic trauma experiment comparing five sampling techniques

Sample pool	Technique ^a				
	1	2	3	4	5
	%T	%T	%T	%T	%T
1	94.0	94.5	93.5	90.5	96.0
2	94.0	95.0	94.5	92.0	95.0
3	94.0	95.5	93.0	93.0	96.0
4	92.5	92.5	92.0	85.0	95.0

All values versus distilled water as 100%T

^aTechnique: (1) No needle, draw sample, air; (2) No needle, draw air, sample; (3) No needle, draw sample, air through needle; (4) Needle, draw sample, air; (5) No needle, draw sample, no air.

These data suggest that technique (5) is superior to the others, including the one previously in use: technique (1). Adoption of technique (5), requiring 4 ml samples taken without an air pad, improves precision at a cost of a 33% increase in the consumption of virus and red blood cells. This increase, the additional consumption of virus in particular, is by no means a factor to be lightly dismissed. Nevertheless, the technique of taking 4 ml samples without an air pad was adopted to reduce error in the series of experiments to determine initial hemolysis kinetics.

The second factor influencing the progress of hemolysis was traced to the plastic beakers used to contain the pool of incubating red blood cells and virus, from which aliquots were taken during the experimental run. Polypropylene disposable beakers had been purchased to facilitate disposal of contaminated equipment at the end of an experimental run. The red blood cell suspensions were pipetted into these beakers which were

then covered and refrigerated from 1 to 16 hours until needed. Although no significant alterations appeared in the blanks, the exposure of the erythrocytes to these beakers caused significant and non-uniform alteration in the response to NDV-induced hemolysis. Substitution of glass beakers greatly relieved this problem.

Kinetics Measurements

The procedure required to determine the initial kinetics of the liberation of hemoglobin was developed, tested and perfected in the preceding experiments. It was found that thermal quenching and centrifugation was insufficient to inhibit hemolysis while the turbidity was being removed for spectrophotometry. A filtration technique was developed which could, in effect, stop the hemolysis within a few seconds and which satisfied the requirements of the procedure for kinetics measurement. Difficulties in the use of the technique were identified and eliminated or reduced in significance. The procedure may now be applied to the problem for which it was perfected.

Experimental

The kinetics measurements which have been the object of this study may be inferred from data relating reaction velocity (rate of hemolysis) to substrate (erythrocyte) concentration. The final series of experiments is an attempt to obtain data which will allow interpretation in kinetic terms. In keeping with previously stated arguments, data relating hemolysis to the passage of time ($\log \%T$ vs. t) are obtained as close to time zero as possible. Experimental limitations, even with the improved technique of sampling, the use of glass beakers to contain the hemolyzing

reaction and the use of the filtration technique, make such measurements somewhat imprecise. This series is a single experimental regimen four times repeated to minimize the effects of the experimental vagary.

A pool of washed red blood cells was taken up with citrate-saline to approximately 10% by volume. The concentration was determined by hemocytometer count and adjusted by further dilution to exactly 10.0% rbc (6×10^8 erythrocytes/ml). This stock preparation was placed in a convoluted flask and kept in suspension by vigorous stirring with a magnetic stirring bar¹. Appropriate aliquots were taken by pipette and transferred to separate 150 ml glass beakers to prepare 35 ml of each of the graded series, 0.5%, 1.0%, 2.0%, 3.0%, 4.0%, 5.0%, 6.0%, 7.0% rbc. These concentrations are stated on the basis of final values after addition of virus; dilution of the 10.0% rbc aliquot must take this into account. Specifically, into each beaker was pipetted:

1.75 ml	10.0% rbc	plus 29.75 ml	citrate-saline	→ 0.5% rbc
3.5 ml	10.0% rbc	plus 28.0 ml	citrate-saline	→ 1.0% rbc
7.0 ml	10.0% rbc	plus 24.5 ml	citrate-saline	→ 2.0% rbc
10.5 ml	10.0% rbc	plus 21.0 ml	citrate-saline	→ 3.0% rbc
14.0 ml	10.0% rbc	plus 17.5 ml	citrate-saline	→ 4.0% rbc
17.5 ml	10.0% rbc	plus 14.0 ml	citrate-saline	→ 5.0% rbc
21.0 ml	10.0% rbc	plus 10.5 ml	citrate-saline	→ 6.0% rbc
24.5 ml	10.0% rbc	plus 7.0 ml	citrate-saline	→ 7.0% rbc.

¹
If foaming is avoided, this procedure is relatively harmless to the blanks and allows more uniform aliquots to be taken for quantitative dilution.

This graded series was prepared in triplicate or in quadruplicate for each repetition of the experiment. The beakers containing the erythrocyte substrate dilutions were tightly covered and refrigerated at 4°C until the hemolytic NDV was prepared.

The hemolytic virus suspension, prepared as usual, was divided into equal portions, one for each concentration of substrate, in separate test tubes or small flasks. These portions were maintained at room temperature until needed. During the course of the experiment one portion of the virus was incubated at 37°C and from it was pipetted all the hemolytic material for a triplicate or quadruplicate determination for one substrate concentration. A fresh portion of hemolytic NDV was incubated for each change of substrate concentration. This procedure was adopted to avoid the irreversible precipitation of hemolytic particles resulting from long exposure of the virus suspension to the 37°C temperature.

A beaker containing the premeasured quantity of rbc substrate was removed from the refrigerator and immersed in the 37°C water bath. The erythrocytes were resuspended by stirring with a Teflon-covered magnetic stirring bar. This pre-incubation period was continued for 3 minutes. During this period a 5 cc disposable syringe was prepared to contain 0.5 ml of citrate-saline. At the end of the 3 minute period of preincubation, the prepared syringe was used to withdraw 4.5 ml of rbc suspension from the beaker. The sample was filtered to prepare a spectrophotometer blank. The added citrate-saline served as a correction factor to adjust for the virus suspension to be added to the remaining substrate.

Using a graduated 5 ml pipet, 3.0 ml of hemolytic NDV suspension from the incubating portion was added to the 27 ml of rbc substrate in the beaker.

At the moment of addition the time-clock was started. Samples of the hemolyzing mixture were taken by syringe and expelled through a filter at 45, 90 and 135 seconds elapsed time. The filtrate was collected directly in 7 mm test tube cuvettes for spectrophotometry. This procedure was repeated for each replicate determination and for every concentration of substrate. At the conclusion of the experiment all the filtrates were assayed using distilled water as 100%T in the Coleman Jr. spectrophotometer.

The Percent Transmittance scale of the Coleman Jr. spectrophotometer is easier to read accurately than the absorbance (optical density) scale. The spectrophotometric assay was, therefore, read in %T, corrected for filter adsorption of hemoglobin (Figure 9) and the corrected values converted to corresponding absorbance. The resulting absorbance vs. elapsed time data are grouped in the 8 tables, Tables 14 - 21, which follow. Each table presents an accumulation of all the data pertaining to a single substrate concentration from all of the experiments performed. For example, Table 14 presents 10 determinations at each of 4 elapsed times collected from 3 experiments in which 0.5% rbc was used. Table 16 presents 13 determinations at each of 4 elapsed times collected from 4 experiments in which 2.0% rbc was used.

Derivation of absorbance-time equations

The data presented in Tables 14 - 21 are an experimental determination of the absorbance (A) as a function of the elapsed time (t); i.e., $A = g(t)$. A more convenient relationship for kinetic interpretation is the reaction velocity (v) as a function of time; i.e., $v = f(t)$. The velocity is

Table 14. Absorbance (A) versus elapsed time for 0.5% rbc (Data accumulated from 3 experiments)

Time seconds	A	Time seconds	A	Time seconds	A	Time seconds	A
0	0.02228	45	0.02228	90	0.01773	135	0.02228
0	0.02687	45	0.02228	90	0.02228	135	0.02228
0	0.02228	45	0.02228	90	0.02687	135	0.02687
0	0.00436	45	0.02228	90	0.01547	135	0.02228
0	0.02228	45	0.01773	90	0.01547	135	0.01773
0	0.01547	45	0.01773	90	0.01773	135	0.02228
0	0.02687	45	0.01773	90	0.02228	135	0.02228
0	0.00877	45	0.01773	90	0.02228	135	0.02228
0	0.01547	45	0.01547	90	0.02687	135	0.01773
0	0.01773	45	0.02228	90	0.02228	135	0.02228

Table 15. Absorbance (A) versus elapsed time for 1.0% rbc (Data accumulated from 3 experiments)

Time seconds	A	Time seconds	A	Time seconds	A	Time seconds	A
0	0.02228	45	0.03152	90	0.03152	135	0.03858
0	0.01773	45	0.02228	90	0.03152	135	0.03621
0	0.02228	45	0.03152	90	0.03152	135	0.03858
0	0.01773	45	0.00877	90	0.01547	135	0.02228
0	0.00436	45	0.00877	90	0.01547	135	0.01773
0	0.01547	45	0.00877	90	0.01547	135	0.02228
0	0.00877	45	0.02228	90	0.01547	135	0.03621
0	0.01547	45	0.02228	90	0.02228	135	0.03621
0	0.01547	45	0.02228	90	0.02228	135	0.03621
0	0.01547	45	0.01773	90	0.01547	135	0.03621

Table 16. Absorbance (A) versus elapsed time for 2.0% rbc (Data accumulated from 4 experiments)

Time seconds	A	Time seconds	A	Time seconds	A	Time seconds	A
0	0.02687	45	0.02228	90	0.03858	135	0.06048
0	0.03152	45	0.03152	90	0.04335	135	0.06048
0	0.03152	45	0.03152	90	0.03858	135	0.05552
0	0.02228	45	0.03152	90	0.01547	135	0.04335
0	0.02228	45	0.02228	90	0.03152	135	0.05552
0	0.02228	45	0.01773	90	0.01773	135	0.05552
0	0.02228	45	0.02228	90	0.02228	135	0.05799
0	0.01773	45	0.02228	90	0.03858	135	0.07058
0	0.01547	45	0.01773	90	0.03152	135	0.06298
0	0.02687	45	0.01773	90	0.02228	135	0.05061
0	0.01547	45	0.03152	90	0.03152	135	0.06803
0	0.02228	45	0.02228	90	0.02228	135	0.06298
0	0.02228	45	0.02228	90	0.03152	135	0.06803

Table 17. Absorbance (A) versus elapsed time for 3.0% rbc (Data accumulated from 3 experiments)

Time seconds	A	Time seconds	A	Time seconds	A	Time seconds	A
0	0.03621	45	0.04335	90	0.05552	135	0.08619
0	0.03858	45	0.03621	90	0.07058	135	0.08884
0	0.03858	45	0.05061	90	0.06048	135	0.08092
0	0.01773	45	0.02687	90	0.05799	135	0.07572
0	0.02228	45	0.02687	90	0.04818	135	0.08355
0	0.01773	45	0.03621	90	0.05799	135	0.08092
0	0.01547	45	0.03858	90	0.05552	135	0.07831
0	0.02687	45	0.02687	90	0.05061	135	0.09151
0	0.02228	45	0.03858	90	0.05799	135	0.09420
0	0.01773	45	0.03152	90	0.05061	135	0.08355

Table 18. Absorbance (A) versus elapsed time for 4.0% rbc (Data accumulated from 4 experiments)

Time seconds	A	Time seconds	A	Time seconds	A	Time seconds	A
0	0.04335	45	0.04335	90	0.06048	135	0.10237
0	0.03152	45	0.04818	90	0.06048	135	0.11070
0	0.03858	45	0.04818	90	0.07058	135	0.11070
0	0.00436	45	0.01773	90	0.04818	135	0.10513
0	0.00436	45	0.02228	90	0.03621	135	0.08619
0	0.01773	45	0.03152	90	0.06803	135	0.07058
0	0.02687	45	0.03152	90	0.07058	135	0.12494
0	0.02228	45	0.04335	90	0.09151	135	0.10513
0	0.02228	45	0.04335	90	0.06550	135	0.11351
0	0.03858	45	0.02687	90	0.06048	135	0.11070
0	0.01547	45	0.03152	90	0.08092	135	0.11919
0	0.03858	45	0.03152	90	0.07572	135	0.10237
0	0.01773	45	0.03621	90	0.06803	135	0.09691

Table 19. Absorbance (A) versus elapsed time for 5.0% rbc (Data accumulated from 4 experiments)

Time seconds	A	Time seconds	A	Time seconds	A	Time seconds	A
0	0.05552	45	0.05552	90	0.09420	135	0.12205
0	0.04818	45	0.05552	90	0.06803	135	0.11634
0	0.04576	45	0.05552	90	0.08884	135	0.10237
0	0.02228	45	0.04335	90	0.08092	135	0.09691
0	0.00436	46	0.03152	90	0.09691	135	0.13371
0	0.03152	45	0.02687	90	0.06298	135	0.11070
0	0.03858	45	0.05552	90	0.10237	135	0.17070
0	0.03858	45	0.05552	90	0.13371	135	0.18709
0	0.03152	45	0.06298	90	0.09691	135	0.14569
0	0.02687	45	0.07058	90	0.10237	135	0.15490
0	0.02228	45	0.05061	90	0.07058	135	0.13668
0	0.02228	45	0.03858	90	0.09151	135	0.13966
0	0.02228	45	0.05061	90	0.07058	135	0.11351

Table 20. Absorbance (A) versus elapsed time for 6.0% rbc (Data accumulated from 4 experiments)

Time seconds	A	Time seconds	A	Time seconds	A	Time seconds	A
0	0.05552	45	0.05799	90	0.08884	135	0.14267
0	0.05061	45	0.07058	90	0.08619	135	0.14267
0	0.04818	45	0.06048	90	0.08619	135	0.13966
0	0.04818	45	0.05552	90	0.10237	135	0.15802
0	0.02228	45	0.03152	90	0.07572	135	0.15802
0	0.02228	45	0.04335	90	0.09151	135	0.13371
0	0.04818	45	0.08092	90	0.12784	135	0.17718
0	0.03858	45	0.06803	90	0.11351	135	0.16115
0	0.04818	45	0.06550	90	0.13371	135	0.20412
0	0.03858	45	0.06803	90	0.11351	135	0.16115
0	0.04818	45	0.06550	90	0.09691	135	0.14874
0	0.04335	45	0.08355	90	0.12494	135	0.14874
0	0.03152	45	0.06048	90	0.11070	135	0.16431

Table 21. Absorbance (A) versus elapsed time for 7.0% rbc (Data accumulated from 4 experiments)

Time seconds							
0	0.06048	45	0.07572	90	0.11070	135	0.14874
0	0.06048	45	0.07572	90	0.11351	135	0.16431
0	0.06803	45	0.07831	90	0.11634	135	0.15490
0	0.02228	45	0.07572	90	0.15181	135	0.16115
0	0.02687	45	0.06803	90	0.09963	135	0.17718
0	0.06298	45	0.05799	90	0.12494	135	0.19723
0	0.04818	45	0.08619	90	0.12205	135	0.20066
0	0.05552	45	0.09151	90	0.14569	135	0.19044
0	0.05552	45	0.07572	90	0.16115	135	0.21112
0	0.04818	45	0.08884	90	0.13966	135	0.14267
0	0.04818	45	0.07058	90	0.13371	135	0.16749
0	0.04657	45	0.08092	90	0.19190	135	0.16749
0	0.04335	45	0.06550	90	0.11351	135	-

related to the absorbance-time curve in that it (v) is defined at the time t' as the slope of the curve $A = g(t)$ at the time t' . The slope of the curve at t' is also the slope of a straight line tangent to the curve at the point $A = g(t')$. The slope of the tangent line and the curve will be the value of v at that point. This value can be obtained from the data in Tables 14-21 by any of several methods, among which are:

(1) Algebraic approximation

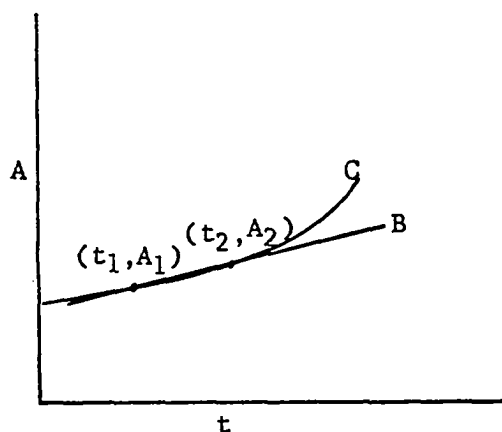


Figure 17. Illustration of algebraic approximation of the tangent to a curve

In Figure 17, smooth curve C represents the experimental curve $A = g(t)$. If a point, say (t_1, A_1) is chosen sufficiently close to (t_2, A_2) , then the line B through (t_1, A_1) , (t_2, A_2) will have a slope m which is approximately v between t_1 and t_2 . As $t_2 - t_1$ approaches zero, the approximation improves.

(2) Mechanical approximation

A mirror or a pair of small glass cylindrical rods can be used to construct a tangent to the curve $A = g(t)$ at a given point. When the curve is reflected smoothly in a mirror held perpendicular to the plane of the curve, the plane of the mirror is normal to the curve at the point of intersection. When the curve appears to continue smoothly through two glass rods laid side by side across it, the adjacent edge of the rods is normal to the curve. In either case the tangent (perpendicular to the normal) to the curve can be constructed through the point of intersection between curve and normal.

(3) The calculus

Differentiation of the equation of the curve $A = g(t)$ provides an equation which represents the slope of the tangent to the curve at any time t . The greatest difficulty in the application of this method lies in the necessity for an equation representing $A = g(t)$.

Methods (1) and (2) are approximations at best. When the curve $A=g(t)$ itself is an approximation ("best fit") to the experimental values, these two methods are subject to still greater departure from accuracy. Furthermore, unless all experimental values are used to establish the curve of "best fit" throughout the range of the independent variable (time), the potential of approximation error increases still further. On the other hand, it is possible to utilize all the experimental data to establish an equation which generates a "best fit" curve. The velocity as a function of time, $v = f(t)$ is obtained immediately by differentiation of that equation.

There are several types of equations which are commonly used to generate curves approximating experimental data. The choice among these is not entirely arbitrary but is dictated by the known facts pertinent to the system from which the data were obtained. In this case the absorbance is known to begin at zero and to always increase to a maximum value at some finite time after hemolysis commences. Under the conditions of the experiments for initial kinetics the curve of $\log \%T$ vs. t is concave downward and has no inflections, oscillations or discontinuities during the time interval observed. This behavior is mathematically rather simple and may be approximated by a polynomial with constant coefficients. Such a polynomial could provide a reasonable basis for interpretation of the mechanism of hemolysis.

A polynomial curve-fitting subroutine was available as part of the software for an IBM 7094 computer facility at Ohio State University, Columbus, Ohio. A computer program was constructed to utilize this subroutine for the purpose of determining the polynomial curves that best fit the data in Tables 14 - 21. This program determined the coefficients for polynomials of order 0 through order 6 which best fit the experimental data. The standard deviation from the mean was calculated for each polynomial. Finally, for each value of $t(0, 45, 90, 135)$ from the data, a new value of the absorbance was calculated using each of the polynomials. For perfect fit of a given polynomial the calculated A must be identical to the experimental A for every time interval.

The standard deviation from the mean is as small as possible for the polynomial expression which best fits the experimental data. For the 8

sets of data (Tables 14 - 21) the computer analysis frequently showed that either a second order or a third order polynomial fit approximately equally well. Evidence of prior experiments (Figure 11b, Figure 13, Figure 14) indicates that the absorbance versus time curve bends in only one direction in the time interval of the last experiments. This unidirectional bending is characteristic of the quadratic equation (second order polynomial): $A = a + bt + ct^2$. Higher orders serve only to allow the polynomial equation to follow the experimental variation. Obviously there is no advantage to following every deviation in the data, such a course of action leads only to confusion. The real system surely follows a curve which under ideal conditions would be most closely approximated by a second order polynomial.

The second order polynomial approximations generated by the computer are listed in Table 22, with their associated standard deviations, for each substrate concentration. Figure 18 is a plot of each of these quadratics on a single graph as an illustration of their relationship. No experimental data are shown in Figure 18. Figures 19 - 22, however, show the data of substrate concentrations 1.0%, 2.0%, 4.0%, and 6.0% (from Tables 15, 16, 18, and 20, respectively), together with the quadratic of best fit.

Interpretation of reaction mechanism

Determination of velocity equations

Figures 19 - 22 show that experimental scatter is still a very significant hindrance to the interpretation of the data. Using the material available, however, it is possible to make some at least tentative statements concerning the early

Figure 18. Composite graph of "best-fit" quadratics as obtained by computer analysis. Points shown are not experimental data.

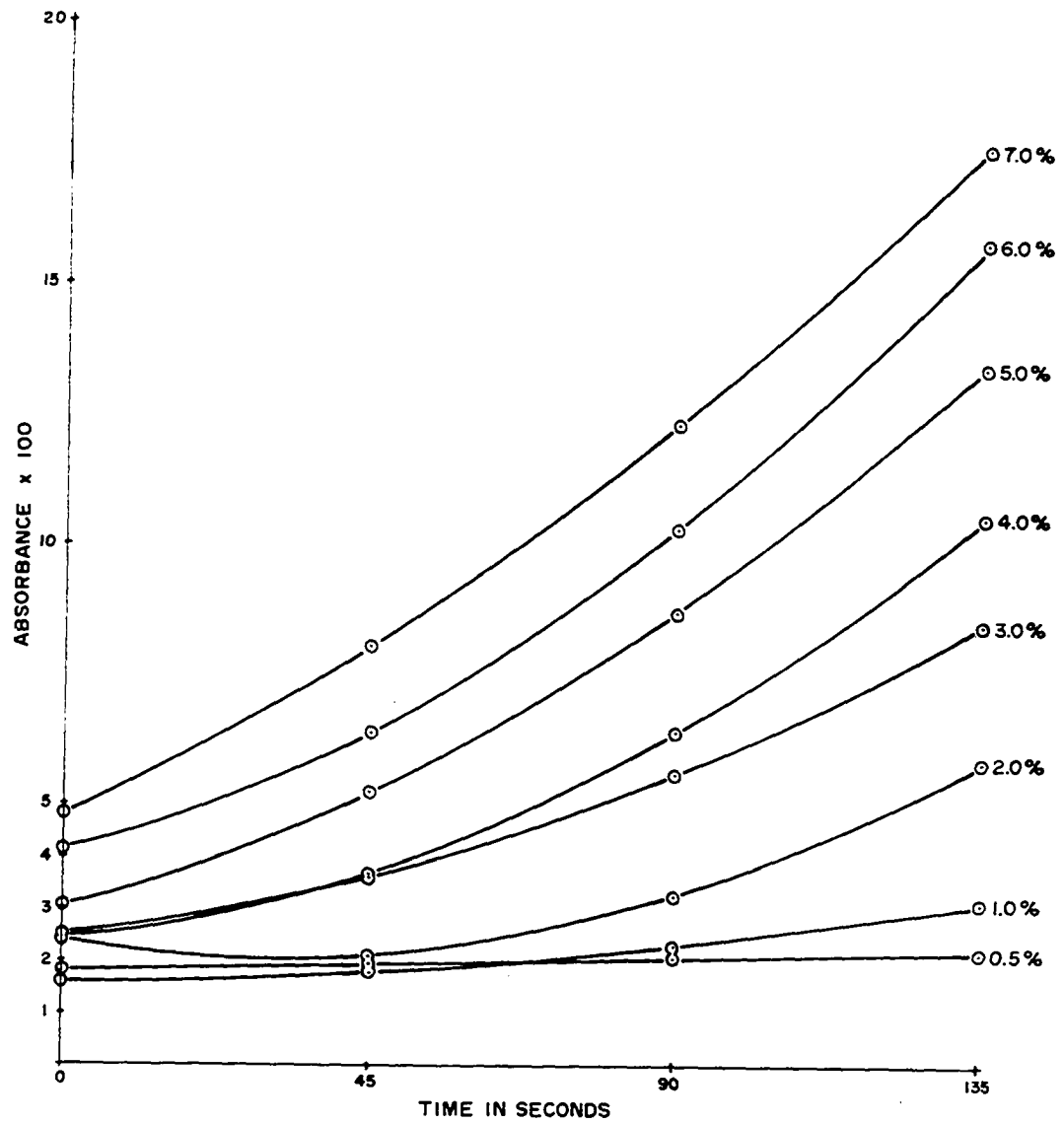


Figure 19. Experimental data from 1.0% rbc (see Table 15),
plotted against "best-fit" quadratic.

Figure 20. Experimental data from 2.0% rbc (see Table 16),
plotted against "best-fit" quadratic.

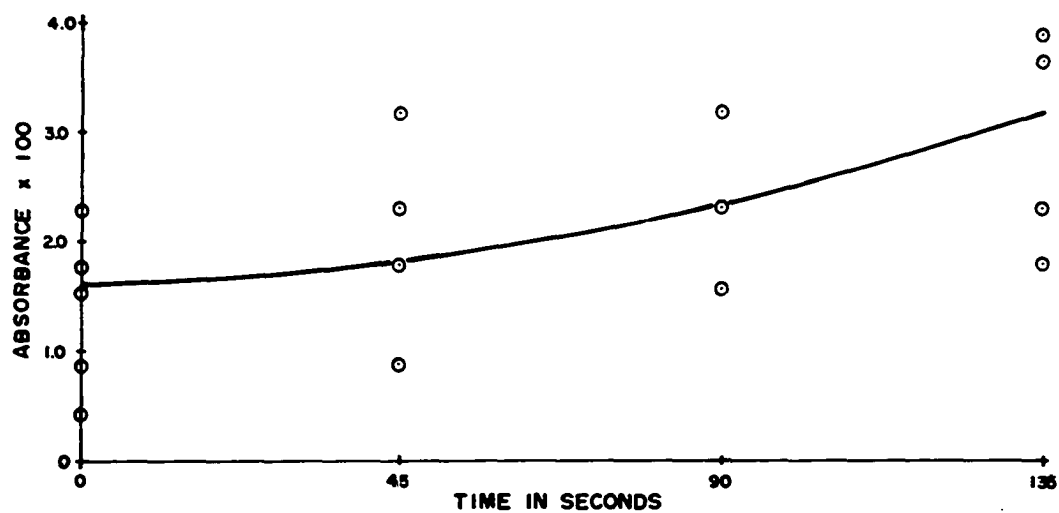


Figure 21. Experimental data from 4.0% rbc (see Table 18), plotted against "best-fit" quadratic.

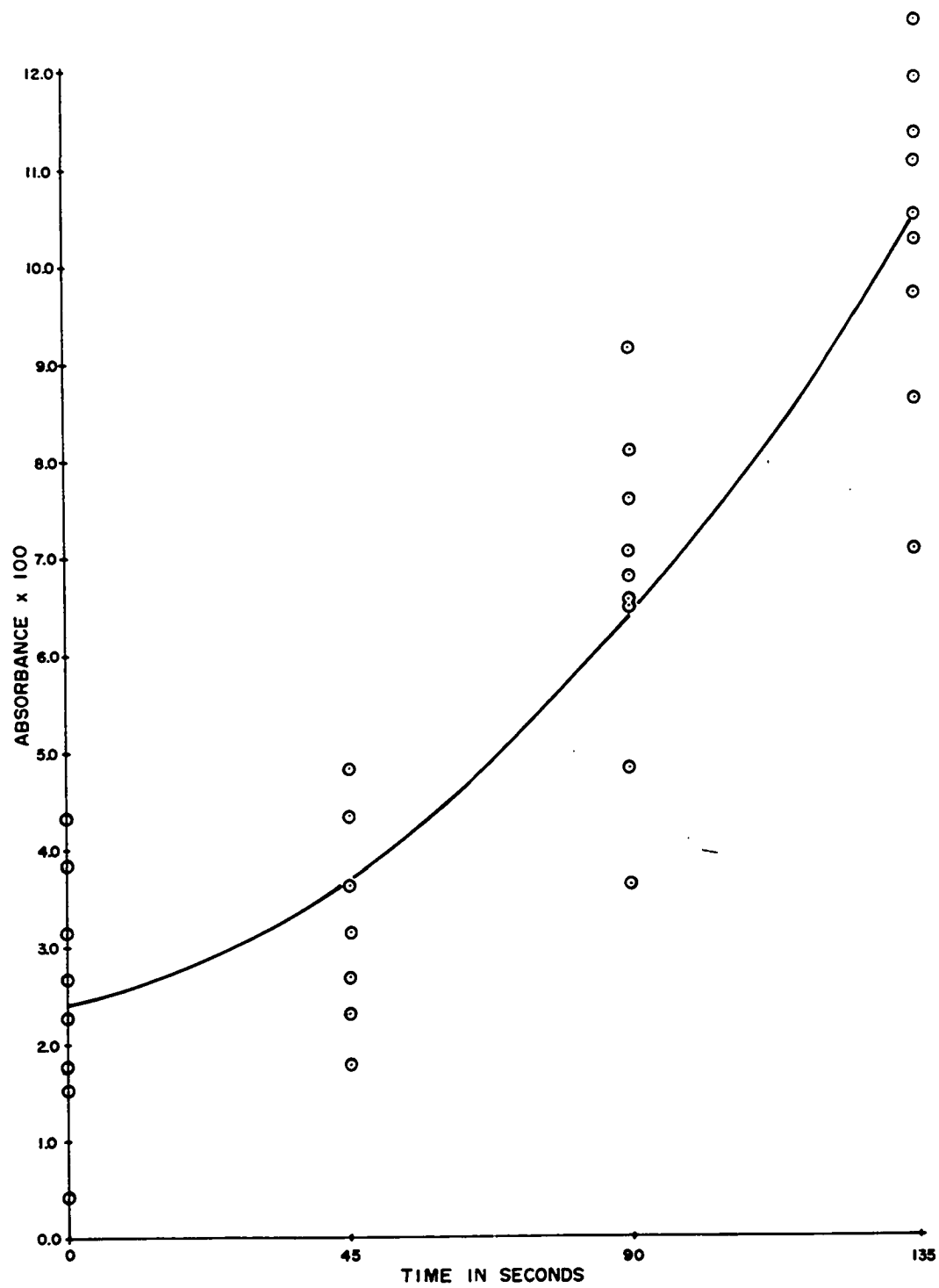
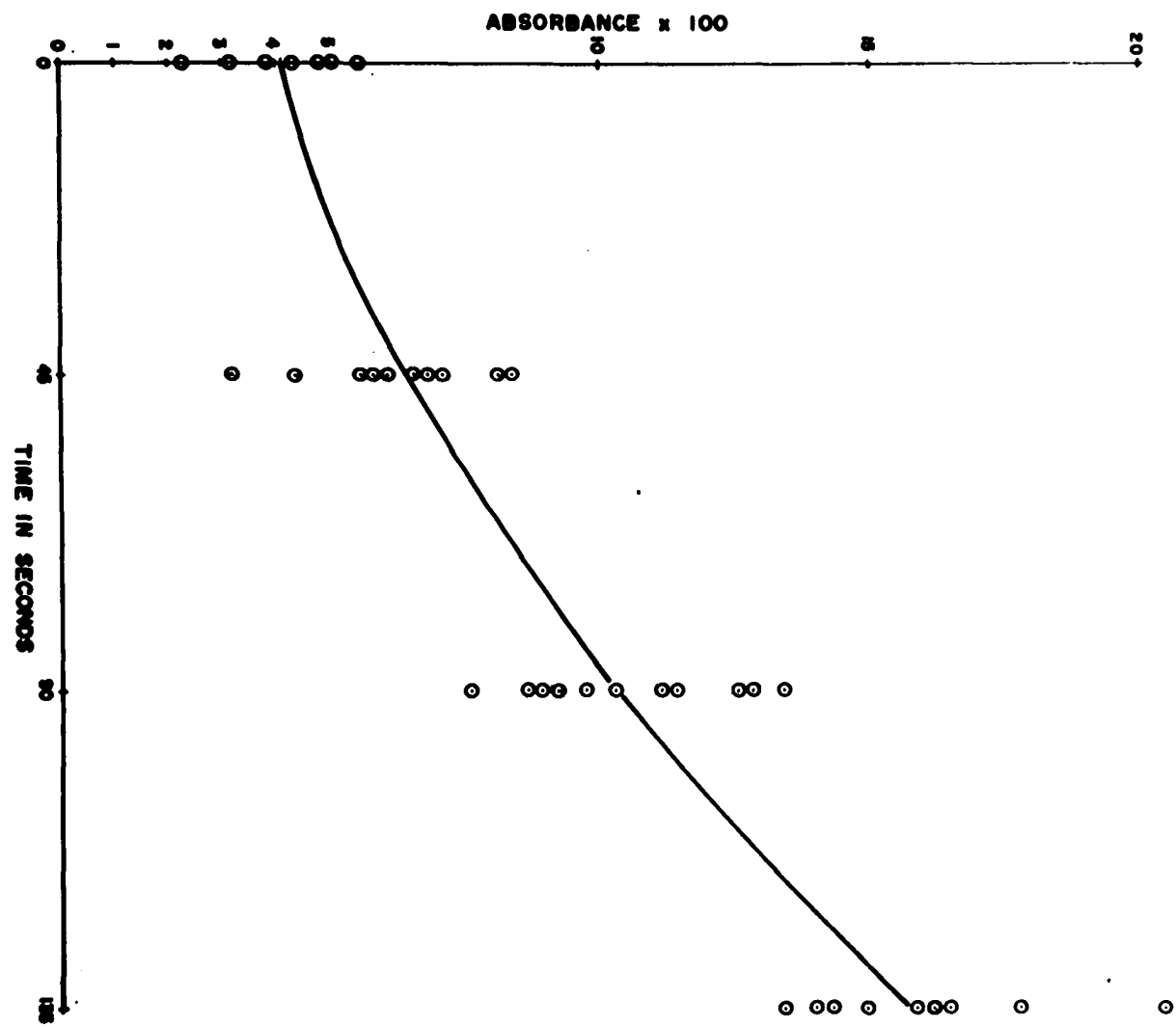


Figure 22. Experimental data from 6.0% rbc (see Table 20),
plotted against "best-fit" quadratic.



kinetics of hemolysis by NDV. If the reaction velocity equations are obtained by differentiation of the second order polynomials of Table 22, it is possible to calculate values for v at any point of time within the interval 0 - 135 seconds. These velocity equations are presented in Table 23. The velocities at $t = 0$ are the parameters of choice, but the data are too imprecise at the earliest time interval to permit meaningful interpretation. An examination of the equations in Table 22 shows that the

Table 22. Computer-generated polynomial equations of best fit for the data of Tables 14 - 21

Substrate concentration	Equation of approximating curve second order polynomial	Standard deviation from mean
0.5%	$A = 0.01825 + 3.712 \times 10^{-5}t - 7.877 \times 10^{-8}t^2$	4.42×10^{-3}
1.0%	$A = 0.01603 + 1.005 \times 10^{-5}t + 7.760 \times 10^{-7}t^2$	7.15×10^{-3}
2.0%	$A = 0.02400 - 2.234 \times 10^{-4}t + 3.543 \times 10^{-6}t^2$	7.02×10^{-3}
3.0%	$A = 0.02515 + 1.467 \times 10^{-4}t + 2.173 \times 10^{-6}t^2$	7.05×10^{-3}
4.0%	$A = 0.02410 + 1.286 \times 10^{-4}t + 3.493 \times 10^{-6}t^2$	1.24×10^{-2}
5.0%	$A = 0.03076 + 3.436 \times 10^{-4}t + 3.112 \times 10^{-6}t^2$	1.82×10^{-2}
6.0%	$A = 0.04134 + 3.209 \times 10^{-4}t + 3.992 \times 10^{-6}t^2$	1.51×10^{-2}
7.0%	$A = 0.04831 + 6.031 \times 10^{-4}t + 2.493 \times 10^{-6}t^2$	1.59×10^{-2}

curves for 0.5% and for 2.0% rbc do not follow a pattern similar to that of the other concentrations. The relationship among these curves is most easily visualized by reference to Figure 18. The erratic behavior of these curves is most pronounced during the interval 0 - 45 seconds. Table 23 continues this evidence of difficulty at times close to zero. The

Table 23. Reaction velocity equations for each concentration of substrate

Substrate concentration	Reaction velocity equation $d(A)/dt = v$
0.5%	$v = 3.712 \times 10^{-5} - 15.75 \times 10^{-8}t$
1.0%	$v = 1.005 \times 10^{-5} + 15.52 \times 10^{-7}t$
2.0%	$v = -2.234 \times 10^{-4} + 7.086 \times 10^{-6}t$
3.0%	$v = 1.467 \times 10^{-4} + 4.346 \times 10^{-6}t$
4.0%	$v = 1.286 \times 10^{-4} + 6.986 \times 10^{-6}t$
5.0%	$v = 3.436 \times 10^{-4} + 6.224 \times 10^{-6}t$
6.0%	$v = 3.209 \times 10^{-4} + 7.984 \times 10^{-6}t$
7.0%	$v = 6.031 \times 10^{-4} + 4.986 \times 10^{-6}t$

velocity of 0.5% rbc commences reasonably but appears to decrease as time passes; while the velocity of 2.0% rbc hemolysis begins at an impossible negative value. Neither of these situations is reasonable for this system. There is good reason to assume that experimental error accounts for this difficulty at times from 0 - 45 seconds. After a time lapse of 60 seconds, however, the reaction has proceeded far enough to provide a consistent pattern to the velocity, yet is still sufficiently close to the initial time that the decrease in substrate concentration and the effect of side reactions are essentially negligible.

Calculation of reaction velocities Substituting $t = 60$ into the equations of Table 23, values for v , S , $1/v$ and $1/S$ are obtained and pre-

sented in Table 24. These data are now in a form suitable for graphical interpretation.

Table 24. Substrate concentration (S) vs. velocity (V) and their reciprocals from Table 23 (at t = 60)

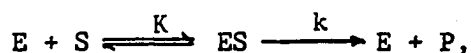
S	$V \times 10^3$ at t=60	1/S	$1/V \times 10^{-3}$ at t=60
$\frac{1}{2}$	0.028	2	36.1
1	0.103	1	9.69
2	0.202	$\frac{1}{2}$	4.96
3	0.407	1/3	2.45
4	0.548	1/4	1.83
5	0.717	1/5	1.40
6	0.800	1/6	1.25
7	0.902	1/7	1.11

Virus = hemolytic enzyme model

Single-site enzyme model

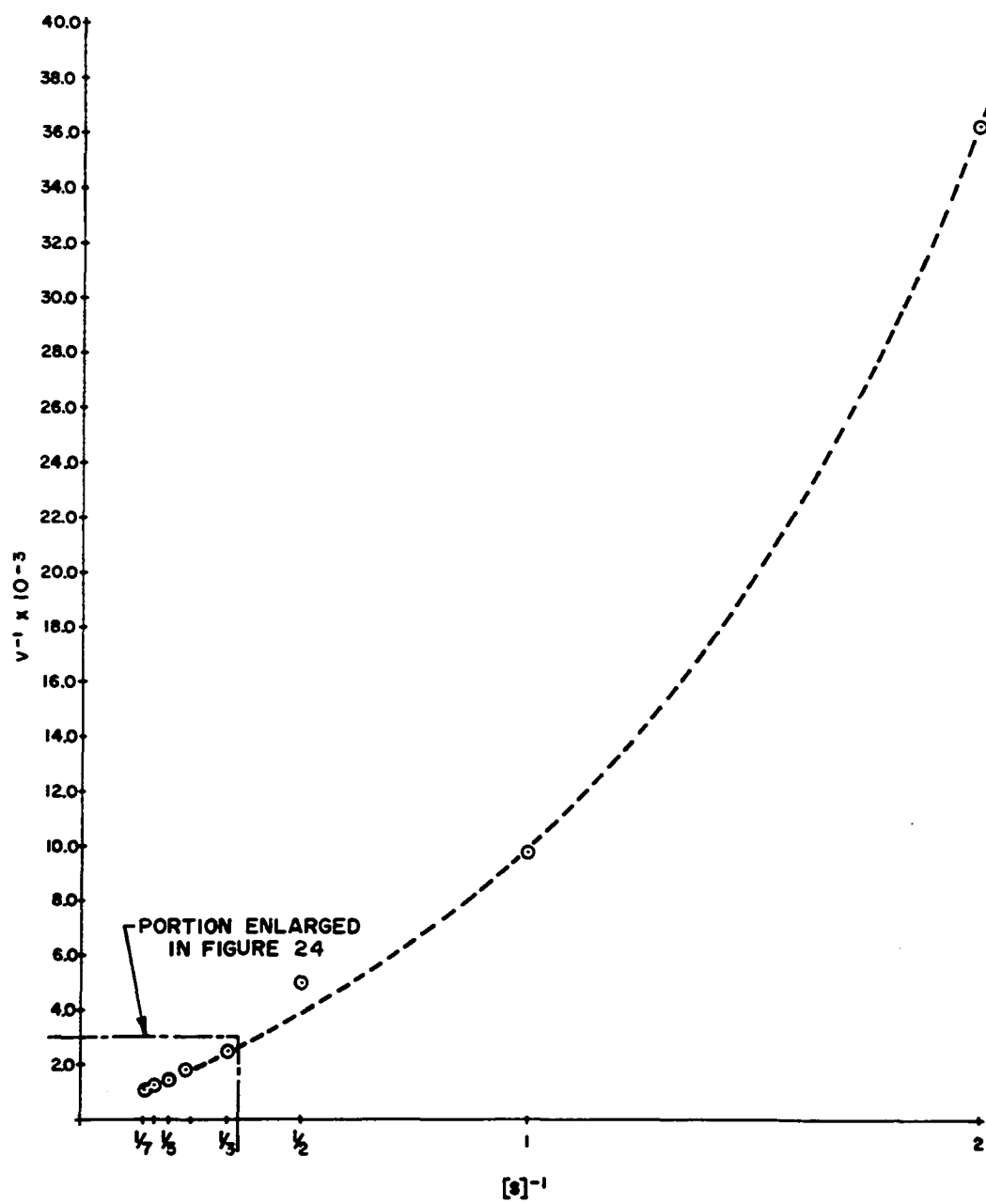
Figure 23 is a Lineweaver-Burk [47]

plot of the data of Table 24. This type of graph yields a linear plot for reaction mechanisms of the type:



where K is an equilibrium constant for the enzyme-substrate complex and k is a rate constant for the breakdown of enzyme-substrate complex to product plus free enzyme. This model may represent an enzyme molecule

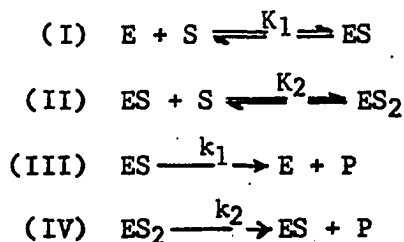
Figure 23. Data of Table 24; Lineweaver-Burk plot: $1/v$ vs. $1/[S]$.



with a single active site capable of combining with substrate. Such a representation is not unique, however, since these kinetics also apply to an enzyme with more than one active site provided that each site is independent of the others. In other words, it is not possible by kinetics measurements alone to distinguish between two moles of enzyme with one active site each or one mole of enzyme with two independent sites each (Reiner [64]). This is not the problem in Figure 23 where the Lineweaver-Burk plot is non-linear. The generalization to a multiple-site enzyme is pertinent, however.

Two-site enzyme model Reiner [64] has shown that curves similar to Figure 23 may be obtained from enzymes which have interacting (non-independent) multiple sites. In nature this is a more realistic model than the independent-site concept. It is reasonable to suppose that the presence of substrate on one site of an enzyme would influence the attachment equilibrium or the conversion rate on a neighboring site. The generalized, n-place enzyme with interacting sites is a model too complicated for a trial-and-error comparison with the experimental data. If the model is limited to two interacting sites, however, it is possible to test the predictions of this model against the experimental results.

Suppose the following mechanism to be a representative two-site enzyme model:



where customarily E = enzyme, S = substrate, ES = one enzyme site occupied with substrate, ES_2 = two enzyme sites occupied with substrate, P = product, K_1 and K_2 are equilibrium constants and k_1 and k_2 are rate constants. In line with the reasoning presented in the Commentary on Approach, the mechanism of the hemolytic event may be explored by assuming that E = hemolytic virus particles, S = susceptible red blood cells, ES = hemolytic virus particles attached to one red blood cell and ES_2 = hemolytic virus particle attached to two red blood cells. The product of the reaction, P, which is an unknown and therefore cannot be measured, may be assumed as a first approximation to be directly related to the liberation of hemoglobin, which is measurable.

By the usual definition of the equilibrium constant:

$$K_1 = [ES]/[E][S], \quad K_2 = [ES_2]/[ES][S]$$

(Brackets are used to denote concentration of the enclosed specie.)

Similarly, the velocity of hemolysis (rate of appearance of hemoglobin) is defined:

$$V = k_1[ES] + k_2[ES_2]$$

and the distribution of hemolytic particles is:

$$[E_T] = [E] + [ES] + [ES_2]$$

where $[E_T]$ represents the total concentration of hemolytic particles in all complex states in the system.

Using the above relationships to express V in terms of measureable quantities:

$$[ES] = K_1 [E][S],$$

$$[ES_2] = K_2 [ES][S] = k_2 K_1 [E][S]^2$$

$$[E_T] = [E] (1 + k_1 [S] + k_1 K_2 [S]^2)$$

$$[E] = [E_T] / (1 + K_1 [S] + K_1 K_2 [S]^2),$$

$$V = k_1 K_1 [E][S] + k_2 K_1 K_2 [E][S]^2$$

$$V = (k_1 K_1 [S] + k_2 K_1 K_2 [S]^2) [E_T] / (1 + K_1 [S] + K_1 K_2 [S]^2).$$

Rearranging V:

$$(V) \quad V = \frac{k_1 K_1 [S] [E_T]}{1 + K_1 [S] + K_1 K_2 [S]^2} + \frac{k_2 K_1 K_2 [E_T] [S]^2}{1 + K_1 [S] + K_1 K_2 [S]^2}$$

and clearing the numerators of $[S]$ and $[S]^2$:

$$(Va) \quad V = \frac{k_1 K_1 [E_T]}{[S]^{-1} + K_1 + K_1 K_2 [S]} + \frac{k_2 K_1 K_2 [E_T]}{[S]^{-2} + K_1 [S]^{-1} + K_1 K_2}.$$

As $[S] \rightarrow \infty$ ($[S]^{-1} \rightarrow 0$), the first term of (Va) approaches zero and the second term approaches $k_2 [E_T]$; i.e., as $[S] \rightarrow \infty$, $V \rightarrow V_{\max}$ and

$$(VI) \quad V_{\max} = k_2 [E_T].$$

The relationship $V_{\max} = k_2 [E_T]$ is reasonable since very high $[S]$ would force nearly all the hemolytic particles into the form ES_2 ; i.e.,

$$[E_T] \rightarrow [ES_2] \quad \text{as} \quad [S] \rightarrow \infty.$$

When $[S]$ is finite and limited as experimental conditions allow, the graph of V^{-1} vs. $[S]^{-1}$ is non-linear due to the appearance of $([S]^{-1})^2$ in equation (VII):

$$(VII) \quad V^{-1} = \frac{([S]^{-1})^2 + K_1 ([S]^{-1}) + K_1 K_2}{E_T (k_1 K_1 [S]^{-1}) + k_2 K_1 K_2}.$$

Furthermore, as $[S]^{-1}$ becomes less than 1, $([S]^{-1})^2$ becomes smaller still and the curve becomes more nearly linear as $[S] \rightarrow \infty$ ($[S]^{-1} \rightarrow 0$). These observations appear to fit the curve of Figure 23 reasonably well. Some further considerations are possible however.

Reiner [64] has suggested the use of a new relationship for further analysis of non-linear Lineweaver-Burk plots. Using Reiner's method, define $r = V/V_{\max}$. From (V) and (VI):

$$r = \frac{(k_1 K_1 [S] + k_2 K_1 K_2 [S]^2) [E_T]}{1 + K_1 [S] + K_1 K_2 [S]^2} \times \frac{1}{k_2 [E_T]}$$

$$r = \frac{k_1 K_1 [S] + k_2 K_1 K_2 [S]^2}{k_2 + k_2 K_1 [S] + k_2 K_1 K_2 [S]^2} .$$

Determine a new variable $r/(1-r)$:

$$1-r = \frac{k_2 + k_2 K_1 [S] + k_2 K_1 K_2 [S]^2 - k_1 K_1 [S] - k_2 K_1 K_2 [S]^2}{k_2 + k_2 K_1 [S] + k_2 K_1 K_2 [S]^2}$$

$$1-r = \frac{k_2 + (k_2 - k_1) K_1 [S]}{k_2 + k_2 K_1 [S] + k_2 K_1 K_2 [S]^2}$$

$$(VIII) \quad r/(1-r) = \frac{k_1 K_1 [S] + k_2 K_1 K_2 [S]^2}{k_2 + (k_2 - k_1) K_1 [S]} .$$

Reducing the numerator of (VIII) to a function linear in $[S]$:

$$(VIIIa) \quad r/(1-r) = \frac{k_1 K_1 + k_2 K_1 K_2 [S]}{k_2 [S]^{-1} + (k_2 - k_1) K_1} .$$

If $[S]$ vs. $r/(1-r)$ were plotted, the relationship would be nearly linear for all $[S] \gg 1$ and asymptotic to the line $r/(1-r) = \frac{k_1}{k_2 - k_1} + \frac{k_2 K_2}{k_2 - k_1} [S]$.

If $k_2 > k_1$, the slope and intercept of the asymptote are positive; if $k_2 < k_1$,

the slope and intercept are negative. If $k_2=k_1$, (VIII) reduces to $r/(1-r) = K_1[S] + K_1K_2[S]^2$ which has no asymptotes. A graph of $r/(1-r)$ versus $[S]$ will be necessary to test these conclusions. To obtain such a graph it will be desirable to extrapolate the curve of Figure 23 to V_{\max} .

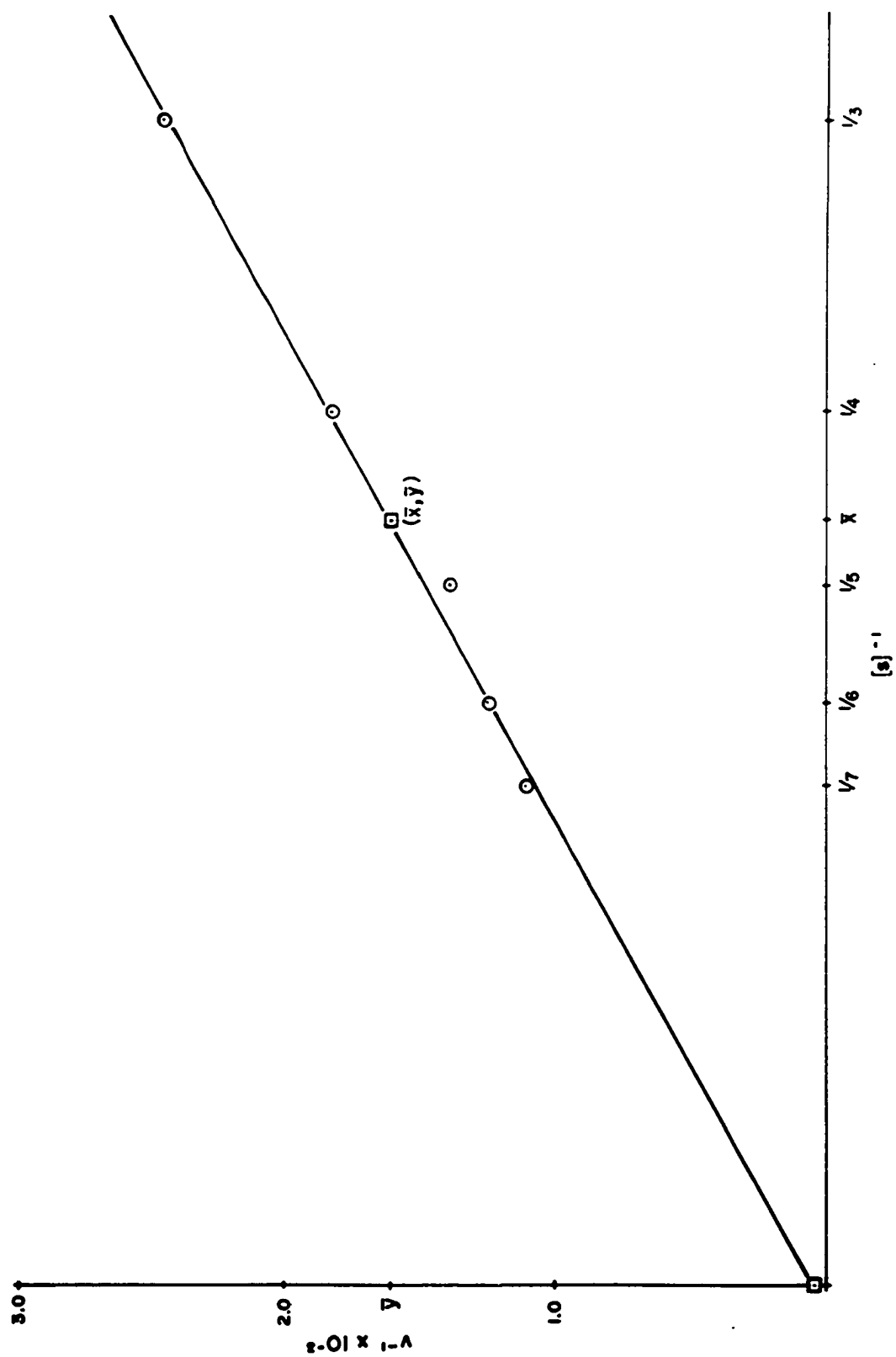
The extrapolation of the curve (Figure 23) to V_{\max} is not as easy as would be the case if the graph were linear. A polynomial curve to fit these data could be computed in a manner identical to that used to generate the curves listed in Table 22. This approach may be misleading, however, due to the large errors involved in the original rate measurements for low $[S]$. On the reciprocal plot of Figure 23 these points of least reliability lie farthest from the origin and strongly influence the shape of the curve. The most reliable velocity calculations were obtained at $S \geq 3$, which points are clustered relatively close to the origin. Through these points a straight line may be drawn with at least as much confidence as a polynomial curve. Such a linear approximation and extrapolation to V_{\max} may yield a value different from that obtained by fitting a curve to the entire graph but the difference will be small compared with the distance of extrapolation.

Figure 24 shows a portion of the graph (Figure 23) of Table 24. The linear approximation is shown and extrapolated to $(V_{\max})^{-1}$ - the V^{-1} -axis intercept. By this method the value for V_{\max} can be calculated:

$$V_{\max} = 1/V_{\max}^{-1} = 1/(0.040 \times 10^{-5})$$

$$(IX) \quad V_{\max} = 25.0 \times 10^{-5} = 0.025 \text{ A units/second.}$$

Figure 24. Enlarged portion of graph; Figure 23. Intercept (V_{\max}^{-1}) estimated by least-squares linear approximation of curve. Coefficient of determination = 0.992, coefficient of correlation = 0.996, intercept = $V_{\max}^{-1} = 0.040 \times 10^3$, $\bar{x} = [\bar{S}]^{-1} = 0.2186$, and $\bar{y} = \bar{v}^{-1} = 1.606 \times 10^3$.



Using this value and the values for V from Table 24 to calculate $r/(1-r)$, the results are shown in Table 25 and plotted in Figure 25.

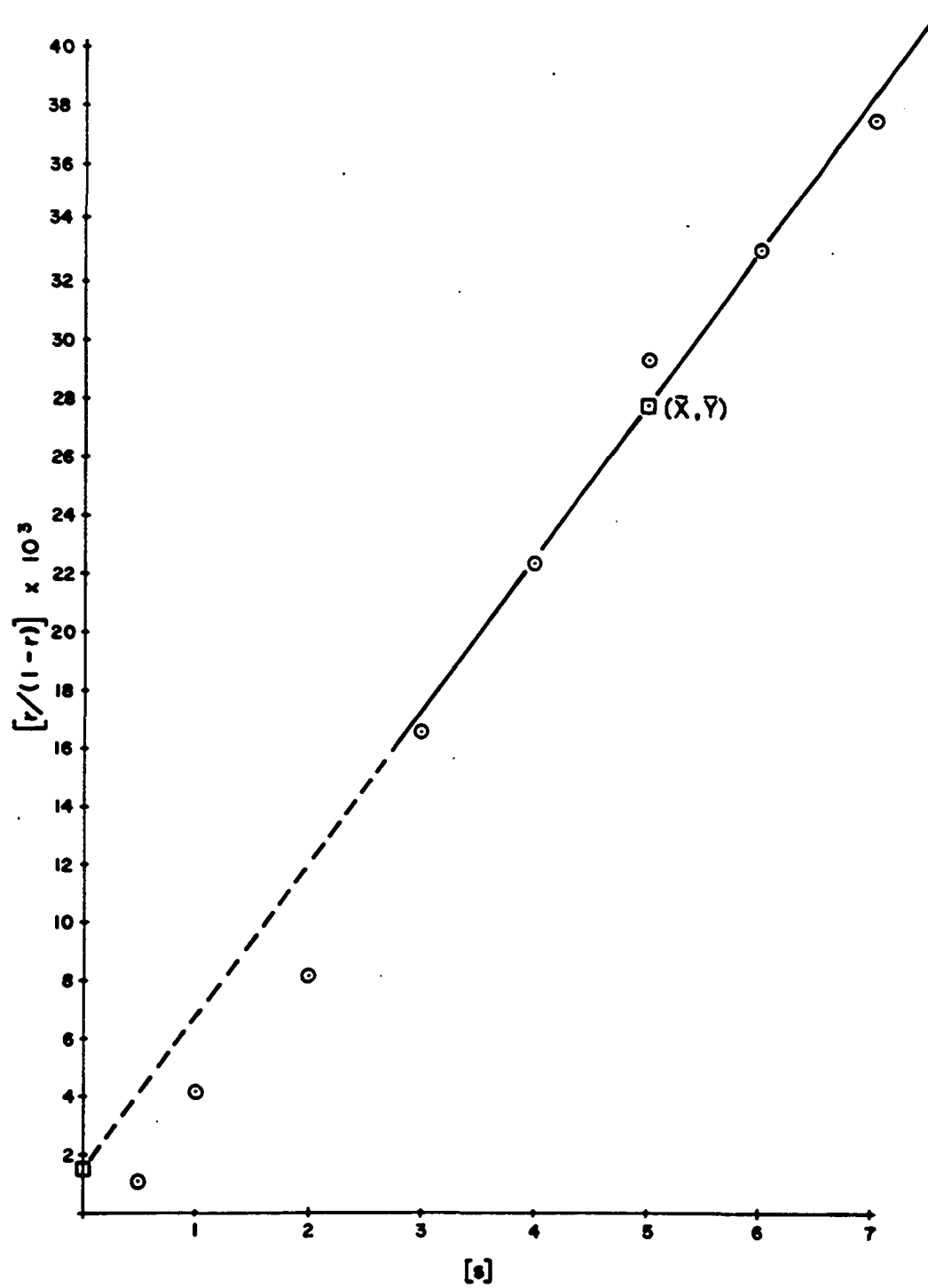
Table 25. Values for $r/(1-r)$ ($= V/(V_{\max} - V)$) at each corresponding value of $[S]$ (% rbc)

$[S]$	$r/(1-r)$	$[S]$	$r/(1-r)$
$\frac{1}{2}$	1.11×10^{-3}	4	22.4×10^{-3}
1	4.15×10^{-3}	5	29.3×10^{-3}
2	8.15×10^{-3}	6	33.1×10^{-3}
3	16.6×10^{-3}	7	37.5×10^{-3}

In Figure 25 the data for large $[S]$ are insufficient to determine an asymptote but they do indicate that $k_2 > k_1$ since the slope (and necessarily the intercept) of the asymptote must be positive. The straight-line approximation to the curve for $[S] > 1$ seems to be confirmed. These manipulations of the rate data appear to support a mechanism of hemolysis which fits the postulated equations (I) - (IV), which is a two-site enzyme model.

It is fairly easy to justify this assumption of a two-site enzyme model as representative of the hemolytic particle of NDV. In the first place, the particle has been shown to react to purification procedures as if it were an intact virus. A particle of viral mass would have physical dimensions adequate to contain several active sites. Wilson [74, 75] using radiation data has suggested that there are approximately 15 hemolytic sites (units) on an NDV particle. However these may be dis-

Figure 25. $r/(1-r)$ versus $[S]$. Least-squares linear approximation to points with $[S] \geq 3$. Coefficient of determination = 0.988, coefficient of correlation = 0.994, intercept = 1.55×10^{-3} , $\bar{x} = [\bar{S}] = 5.0$, and $\bar{y} = \overline{r/(1-r)} = 27.8 \times 10^{-3}$.



tributed on the surface of the virus, it is difficult to imagine that the virus could associate with more than two substrate "molecules" (intact red blood cells) at one time. The hemagglutination reaction serves as a precedent since it is widely accepted as the consequence of virus particles attaching to two erythrocytes simultaneously. The well-established relationship between hemagglutination and hemolysis suggests that hemolysis may also proceed by one- or two-site binding of red blood cells. The experimental data, supported by these arguments, although by no means conclusive are at least a credible case for a bivalent (two-site) mechanism for hemolysis by Newcastle disease virus.

DISCUSSION

It was easy to observe in reviewing the literature that research workers have met with very little success in their efforts to identify the hemolysins present in (or on) some of the myxoviruses. A principle factor contributing to this difficulty has been the inability to isolate the hemolysin from the bulk of the virus particle. It was appropriate, therefore, to consider some new approach to the identification of the hemolysin; an approach based upon those properties of the hemolytic system that are known and can be measured.

The choice of a kinetics approach was almost obvious and was particularly applicable to the system here being investigated since the action of the hemolytic viruses is generally considered to be enzymatic. The kinetic behavior of an enzyme, by the very fact that it is a catalyst, is one of its most significant characteristics. Moreover, the temporal progress of hemolysis in this system is a property that can be readily observed and, at least in principle, measured. In actual practice, however, the experimental data were difficult to obtain with precision; hence the kinetics interpretation was less detailed than was desired. Nevertheless, the goals of this experimental program were at least partially realized in that it was possible to propose a mechanistic model for the interaction of hemolytic NDV and erythrocytes.

When the entire virus particle was considered to be one giant enzyme, i.e., virus concentration = total enzyme concentration, the hemolytic attack appeared to be that of a divalent enzyme. This could be interpreted

as two hemolytic patches or sites, each containing one or more hemolysins, on the surface of the virus. This interpretation is particularly attractive in the light of the known close relationship between HA and hemolysis. It is tempting to speculate that the hemolytic site is intimately associated with the hemagglutinating unit in both a molecular and a topographic sense. The stress of freeze-thaw, lyophilization, dialysis and other techniques might be viewed as causing a minor rearrangement of molecular folding in the regions of the hemagglutinating units which causes the hemolysins to be exposed. The agglutinating attachment of the virus to the surface of a red blood cell would then bring vulnerable structures in the erythrocyte stroma into close proximity of the newly exposed active site. Catalysis would then accelerate some alteration in a vulnerable structure of the red blood cell and hemolysis would follow. Any condition which prevents hemagglutinating attachment of the virus would also prevent hemolysis by denying the active site on the virus the close proximity to the red cell surface that it requires.

In both prospect and retrospect it is somewhat surprising that the assumptions virus = enzyme, red blood cell = substrate and hemolyzing red blood cell = product allowed any meaningful interpretation of the data. These are obviously very gross simplifications of complex participants in a progressive reaction. The success of this model is gratifying not in the interpretation at hand but in the recognition of how much more could be learned by this method with more precise spectrophotometric measurements and a more detailed knowledge of the reaction participants. Two observations resulting from the kinetics research just presented may

provide new opportunities to better understand the nature of the hemolytic particle.

When the virus-red blood cell attachment-elution process was studied to determine the duration of hemolytic particle attachment to an erythrocyte, no change in the infective titer of the supernatant was observed. The hemolytic particle cannot be separated from the infective particle by physical methods such as ultracentrifugation. The filtration technique may prove to be an effective means to study the infectivity-hemolytic activity relationship in the presence of erythrocytes. Whether or not there is any distinction between infectivity and hemolytic activity in this system, the more exacting information that could be provided by the filtration technique would be a definite contribution to the study of viral hemolysins.

Perhaps the method utilized in this research to prepare hemolytic virus would provide an opportunity to reopen the search for the substrate of NDV hemolysin. Those virus particles which adsorb onto the infected allantoic fluid precipitate which forms during slow thawing are much more active hemolytically than the particles remaining in the supernatant, although the infective titers are approximately equal. Is there some identifiable specie or molecular configuration in this precipitate that specifically adsorbs hemolytic particles? Would electron microscopy reveal structural differences between the particles associated with the precipitate and those remaining in the decantate? These questions and many more remain unanswered and await future efforts to invest the measurement of the kinetics of the hemolysin of Newcastle disease virus with greater certainty.

SUMMARY

1. No reduction in infectivity titer of the supernatant was observed during the first three minutes of hemolytic virus-erythrocyte interaction at 37°C. This observation was attributed to two possible alternatives: (a) hemolytic particles and infective particles are not identical, or (b) infective particle-erythrocyte attachment-elution is a rapid equilibrium in which the fraction of virus attached to the red blood cells at any given moment is less than the $\pm 40\%$ sensitivity of the infectivity titration.

2. The classical method for stopping hemolysis, thermal quench, centrifugation and decantation, was found to be inadequate for initial hemolysis measurements. Even at 0°C, hemolysis once initiated can continue appreciably during the centrifugation and decantation procedure.

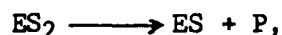
3. A rapid filtration procedure was developed which could in less than 5 seconds remove all erythrocytes from suspensions up to 7% in concentration (42×10^7 red blood cells/ml). The filtration procedure was shown to be reproducible, repeatable, quantitative and well-suited to the study of the initial kinetics of hemolysis. The superiority of this technique over the quench-centrifugation procedure was demonstrated.

4. The loss of hemoglobin, expressed as spectrophotometric absorbance or log %T, resulting from NDV-induced hemolysis was shown to follow a sigmoid relationship with time. The equivalence of absorbance vs. time and percent hemolysis vs. time was demonstrated.

5. Rabbit erythrocytes were shown to be immune to hemolysis by NDV as had been previously reported in the literature.

6. The hemolysis rate was studied as a function of red blood cell concentration during the time interval 0-135 seconds. Best-fit quadratic equations were determined for the absorbance vs. time curve at each substrate concentration. The hemolysis rate functions were obtained as the derivatives of the best-fit quadratic equations.

7. A Lineweaver-Burk plot of (hemolysis rate)⁻¹ vs. (red blood cell concentration)⁻¹ was non-linear. A non-independent, two-site enzyme model for virus action:



where E = virus, S = erythrocyte, P = hemolyzing red blood cell, was shown to fit the experimental data reasonably well. An interpretation of this model in terms of hemolytic virus-red blood cell interaction was presented and discussed.

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